Study Title
The Response of the TRPV1 Receptor to
Capsaicinoids in Mammals

Company Name
Security Equipment Corporation

Product Identification
Frontiersman Bear Attack Deterrent II
EPA File Symbol: 72265-U
(2% Capsaicin and Related Capsaicinoids)

Data Requirement
Pesticide Assessment Guidelines
Subdivision G: Product Performance
OCSPP Guideline Number N.A.
40 CFR § 158.2070

Authors
Caterina, M.J., et al
Pogorzala, L.A., et al
Mishra, S.K., et al

Volume Number
7 of 9

Completion Date
February 14, 2020

Compilation of Public Literature by
RegWest Company, LLC
8209 West 20th Street, Suite B
Greeley, CO 80634-4699

File Name
ST.EFF-TRPV1

Statement of No Data Confidentiality Claims

No information contained in this volume is claimed as confidential on the basis of its falling within the scope of FIFRA Section (10)(d)(1)(A), (B) or (C).

Company:	RegWest Company, LLC for Security Equipment Corporation
Signature:	
Company Agent:	Kim Davis
Title:	Consultant/Agent
Date:	February 14, 2020

Good Laboratory Practice Statement

This report is a compilation of public literature that the applicant did not sponsor or conduct and therefore contains no data to which the requirements of 40 CFR Part 160 apply*.

Submitter:	RegWest Company, LLC for Security Equipment Corporation
Signature:	
Company Agent:	Kim Davis
Title:	Consultant/Agent
Date:	February 14, 2020

Sponsor:	RegWest Company, LLC for Security Equipment Corporation
Signature:	
Company Agent:	Kim Davis
Title:	Consultant/Agent
Date:	February 14, 2020

Study Director*: Not Applicable – There was no study director; see explanation above.

Table of Contents

The Response of the TRPV1 Receptor to Capsaicinoids in Mammals

Introduction

Security Equipment Corporation desires to expand the label claims on its bear attack deterrent products to include large cats such as mountain lions, cougars, pumas, bobcats, lynxes and all other large cats.

The efficacy of pepper spray against bears, dogs and humans has been well established. The mechanism of action is via Capsaicinoids binding to the TRPV1 receptor that modulates the chemical signal into nerve impulses of nociception (pain), experienced as burning heat. The TRPV1 receptor is critical to thermoregulation in all mammals and is ubiquitously expressed in sensory afferent neurons and dorsal root ganglia. The function, prevalence and commensurate binding of identical ligands of this receptor across the entire mammalian class indicates that Capsaicinoids will have efficacy against large cats in the same way it does against bears, dogs and humans.

In lieu of specific empirical testing against large cats that would be difficult, costly, time-consumptive, dangerous and possibly illegal, it is reasonable to conclude that inclusion of large cats on Security Equipment Corporations' product labeling can be supported by submission of peer-review academic papers (articles) demonstrating the TRPV1 receptor is indeed ubiquitously expressed in all mammals.

As examples, in The capsaicin receptor: a heat-activated ion channel in the pain pathway by Michael J. Caterina, et al:

"In mammals, exposure of nociceptor terminals to capsaicin leads initially to excitation of the neuron and the consequent perception of pain and local release of inflammatory mediators…"

"This receptor is also a thermal sensor that is strongly activated when ambient temperatures are elevated to a range known to elicit pain in humans or pain associated behaviors in animals…"

"A mammalian cell expression cloning strategy was devised…"

In The cellular code for mammalian thermosensation by Leah A. Pogorzala, et al:

"… The ability of mammals to sense the temperature in their environment relies primarily on specialized somatosensory neurons in the trigeminal and dorsal root ganglia…"

"… A major advance in the understanding of mammalian temperature sensation came with the characterization of TRPV1 as a high temperature and capsaicin gated-channel that is expressed in heat sensitive DRG neurons…"

And in TRPV1-lineage neurons are required for thermal sensation by Santosh KI. Mishra, et al:

"… the receptor for capsaicin (the 'hot' compound from chili peppers), TRPV1, is expressed in somatosensory neurons and thought to be the major mammalian sensor of noxious heat…"

"… In this study, we used a molecular genetic approach to generate mice lacking all neurons in the TRPV1 lineage. Notably, these animals display no responses to thermal stimuli…"

Article Abstracts

The capsaicin receptor: a heat-activated ion channel in the pain pathway by Michael J. Caterina, et al:
Capsaicin, the main pungent ingredient in 'hot' chili peppers, elicits a sensation of burning pain by selectively activating sensory neurons that convey information about noxious stimuli to the central nervous system. We have used an expression cloning strategy based on calcium influx to isolate a functional cDNA encoding a capsaicin receptor from sensory neurons. This receptor is a non-selective cation channel that is structurally related to members of the TRP family of ion channels. The cloned capsaicin receptor is also activated by increases in temperature in the noxious range, suggesting that it functions as a transducer of painful thermal stimuli *in vivo*.

The cellular code for mammalian thermosensation by Leah A. Pogorzala, et al:
Mammalian somatosensory neurons respond to thermal stimuli allowing animals to reliably discriminate hot from cold and select their preferred environments. We previously generated mice that are completely insensitive to temperatures from noxious cold to painful heat (-5 to 55ºC) by ablating several different classes of nociceptor early in development. Here we have adopted a selective ablation strategy in adult mice to dissect this phenotype and thereby demonstrated that separate populations of molecularly defined neurons respond to hot and cold. TRPV1-expressing neurons are responsible for all behavioral responses to temperatures between 40 and 50ºC, while TRPM8-neurons are required for cold aversion. We also show that more extreme cold and heat activate additional populations of nociceptors including cells expressing Mrgprd. Thus, although eliminating Mrgprd-neurons alone does not affect behavioral response to temperature, when combined with ablation of TRPV1- or TRPM8-cells, it significantly decreases responses to extreme heat and cold respectively. Notably, ablation of TRPM8-neurons distorts responses to preferred temperatures suggesting that the pleasant thermal sensation of warmth my in fact just reflect reduced aversive-input from TRPM8 and TRPV1-neurons. As predicted by the hypothesis, mice lacking both these classes of thermosensor exhibited neiher aversive nor attractive responses to temperatures between 10 and 50ºC. Taken together these results provide a simple cellular basis for mammalian thermosensation whereby two molecularly defined classes of sensory neuros detect and encode both attractive and aversive cues.

TRPV1-lineaage neurons are required for thermal sensation by Santosh K Mishra, et al:
The ion-channel TRPV1 is believed to be a major sensor of noxious heat, but surprisingly animals lacking TRPV1 still display marked responses to elevated temperature. In this study, we explored the role of TRPV1-expressing neurons in somatosensation by generating mice wherein this lineage of cells was selectively labelled or ablated. Our data show that TRPV1 is an embryonic marker of many nociceptors including all TRPV1- and TRPM8-neurons as well as many Mrg-expressing neurons. Mutant mice lacking these cells are completely insensitive to hot or cold but in marked contrast retain normal touch and mechanical pain sensation. These animals also exhibit defective body temperature control and lose both itch and pain reactions to potent chemical mediators. Together with previous cell ablation studies, our results define and delimit the roles of TRPV1- and TRPM8-neurons the thermosensation, thermoregulation and nociception, thus significantly extending the concept of labelled lines in somatosensory coding.

The capsaicin receptor: a heat-activated ion channel in the pain pathway

Michael J. Caterina*, Mark A. Schumacher†‖, Makoto Tominaga*‖, Tobias A. Rosen*, Jon D. Levine‡ & David Julius*

*Departments of * Cellular and Molecular Pharmacology, † Anesthesia, and ‡ Medicine, University of California, San Francisco, California 94143-0450, USA*
‖ These authors contributed equally to this study.

Capsaicin, the main pungent ingredient in 'hot' chilli peppers, elicits a sensation of burning pain by selectively activating sensory neurons that convey information about noxious stimuli to the central nervous system. We have used an expression cloning strategy based on calcium influx to isolate a functional cDNA encoding a capsaicin receptor from sensory neurons. This receptor is a non-selective cation channel that is structurally related to members of the TRP family of ion channels. The cloned capsaicin receptor is also activated by increases in temperature in the noxious range, suggesting that it functions as a transducer of painful thermal stimuli *in vivo*.

Pain is initiated when the peripheral terminals of a subgroup of sensory neurons are activated by noxious chemical, mechanical or thermal stimuli. These neurons, called nociceptors, transmit information regarding tissue damage to pain-processing centres in the spinal cord and brain[1]. Nociceptors are characterized, in part, by their sensitivity to capsaicin, a natural product of capsicum peppers that is the active ingredient of many 'hot' and spicy foods. In mammals, exposure of nociceptor terminals to capsaicin leads initially to excitation of the neuron and the consequent perception of pain and local release of inflammatory mediators. With prolonged exposure, nociceptor terminals become insensitive to capsaicin, as well as to other noxious stimuli[2]. This latter phenomenon of nociceptor desensitization underlies the seemingly paradoxical use of capsaicin as an analgesic agent in the treatment of painful disorders ranging from viral and diabetic neuropathies to rheumatoid arthritis[3,4]. Some of this decreased sensitivity to noxious stimuli may result from reversible changes in the nociceptor, but the long-term loss of responsiveness can be explained by death of the nociceptor or destruction of its peripheral terminals following exposure to capsaicin[2,5].

The cellular specificity of capsaicin action and its ability to evoke the sensation of burning pain have led to speculation that the target of capsaicin action plays an important physiological role in the detection of painful stimuli. Indeed, capsaicin may elicit the perception of pain by mimicking the actions of a physiological stimulus or an endogenous ligand produced during tissue injury[6]. Although the excitatory and neurotoxic properties of capsaicin have been used extensively to define and study nociceptive neurons, its precise mechanism of action has remained elusive. Electrophysiological[7,8] and biochemical[9] studies have shown that capsaicin excites nociceptors by increasing the permeability of the plasma membrane to cations, but the molecular mechanism underlying this phenomenon is unclear. Proposed models range from the direct perturbation of membrane lipids by hydrophobic capsaicin molecules[10] to the activation of a specific receptor on or within sensory neurons[6]. Because capsaicin derivatives show structure–function relationships and evoke responses in a dose-dependent manner[11,12], the existence of a receptor site represents the most likely mechanism. This model has been strengthened by the development of capsazepine, a competitive capsaicin antagonist[13], and by the discovery of resiniferatoxin, an extremely potent capsaicin analogue from *Euphorbia* plants that mimics the cellular actions of capsaicin[14,15]. The potency of resiniferatoxin at nanomolar quantities has led to its use as a high-affinity radioligand to visualize saturable, capsaicin- and capsazepine-sensitive binding sites on nociceptors[16].

A more detailed understanding of the molecular nature of capsaicin action and its relationship to endogenous pain signalling mechanisms might be obtained through the cloning of a gene encoding a capsaicin receptor. To achieve this we used a functional screening assay to isolate a cDNA clone that reconstitutes capsaicin responsiveness in non-neuronal cells. The deduced amino-acid sequence of this clone demonstrates that the capsaicin receptor is an integral membrane protein with homology to a family of putative store-operated calcium channels. The cloned receptor seems to be

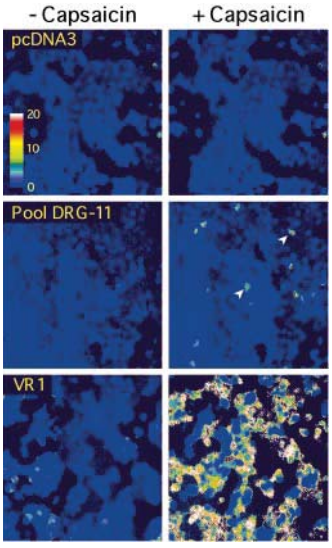


Figure 1 Expression cloning of a capsaicin receptor using calcium imaging. HEK293 cells transiently transfected with pools of clones from a rodent dorsal root ganglion (DRG) cDNA library were subjected to microscopic fluorescent calcium imaging before (left) and during (right) treatment with 3 μM capsaicin. Cells transfected with vector alone (pCDNA3; top) exhibited no response to capsaicin. Between 1% and 5% of cells transfected with pool 11 exhibited marked increases in cytoplasmic calcium (middle, arrowheads). This pool was iteratively subdivided and reassayed until a single positive clone (VR1) was isolated (bottom). Elevated relative calcium concentrations are indicated by an increased ratio of Fura-2 emission at 340 versus 380 nm wavelength excitation (see colour bar).

expressed exclusively by small-diameter neurons within sensory ganglia, providing a definitive molecular explanation for the remarkable selectivity of capsaicin action. This receptor is also a thermal sensor that is strongly activated when ambient temperatures are elevated to a range known to elicit pain in humans or pain-associated behaviours in animals. Thus capsaicin elicits burning sensations through the activation of a heat-gated ion channel that is likely to contribute to the detection of painful thermal stimuli *in vivo*.

Expression cloning of receptor cDNA

The lack of specific information regarding the molecular structure of capsaicin receptors prompted us to adopt a functional screening strategy for isolating candidate cDNA clones. A mammalian cell expression cloning strategy was devised on the basis of the ability of capsaicin to trigger robust calcium influx into sensory neurons *in vitro*[9,17]. We reasoned that a capsaicin receptor-encoding cDNA might confer upon non-neuronal cells a similar ability to undergo increases in intracellular free calcium upon exposure to capsaicin, assuming that capsaicin acts at a proteinaceous site and that a single cDNA can confer sensitivity to capsaicin in a heterologous context. Because capsaicin responsiveness seems to be confined to nociceptive neurons with cell bodies that reside within sensory ganglia[5], a cDNA library was constructed from dorsal root ganglion-derived messenger RNA. This library was subdivided into pools of approximately 16,000 clones, and each pool was transiently transfected into human embryonic kidney-derived HEK293 cells. Transfected cells were then loaded with the fluorescent calcium-sensitive dye Fura-2 (ref. 18), and microscopically examined for capsaicin-evoked changes in intracellular calcium levels. A positive pool was identified (Fig. 1, middle) and iteratively subdivided and reassayed. In this way, an individual clone containing a 3-kilobase (kb) cDNA insert was obtained that, by itself, conferred capsaicin (Fig. 1, bottom) or resiniferatoxin (not shown) sensitivity to transfected HEK293 cells. Because a vanilloid moiety constitutes an essential chemical component of capsaicin and resiniferatoxin structures, the proposed site of action of these compounds is more generally referred to as the vanilloid receptor[16]. Accordingly, we have named the newly cloned cDNA VR1, for vanilloid receptor subtype 1.

VR1 and vanilloid receptor pharmacology

To compare the pharmacological properties of the cloned receptor to those of native vanilloid sites in sensory ganglia, we expressed VR1 in *Xenopus* oocytes and used whole-cell voltage-clamp analysis to quantitatively examine the electrophysiological responses to a variety of vanilloid agonists and antagonists. At negative holding potentials, exposure to capsaicin or resiniferatoxin produced dose-dependent inward current responses in VR1-expressing oocytes, but not in water-injected control cells (Fig. 2a). As observed in sensory neurons[19,20], capsaicin-evoked current responses returned rapidly to baseline after agonist removal, whereas resiniferatoxin responses often failed to recover, even after a prolonged washout period. Half-maximal effective concentrations for these agonists were within an order of magnitude of those reported for native vanilloid receptors[8,13], with resiniferatoxin being approximately 20-fold more potent than capsaicin (EC_{50} = 39.1 nM and 711.9 nM,

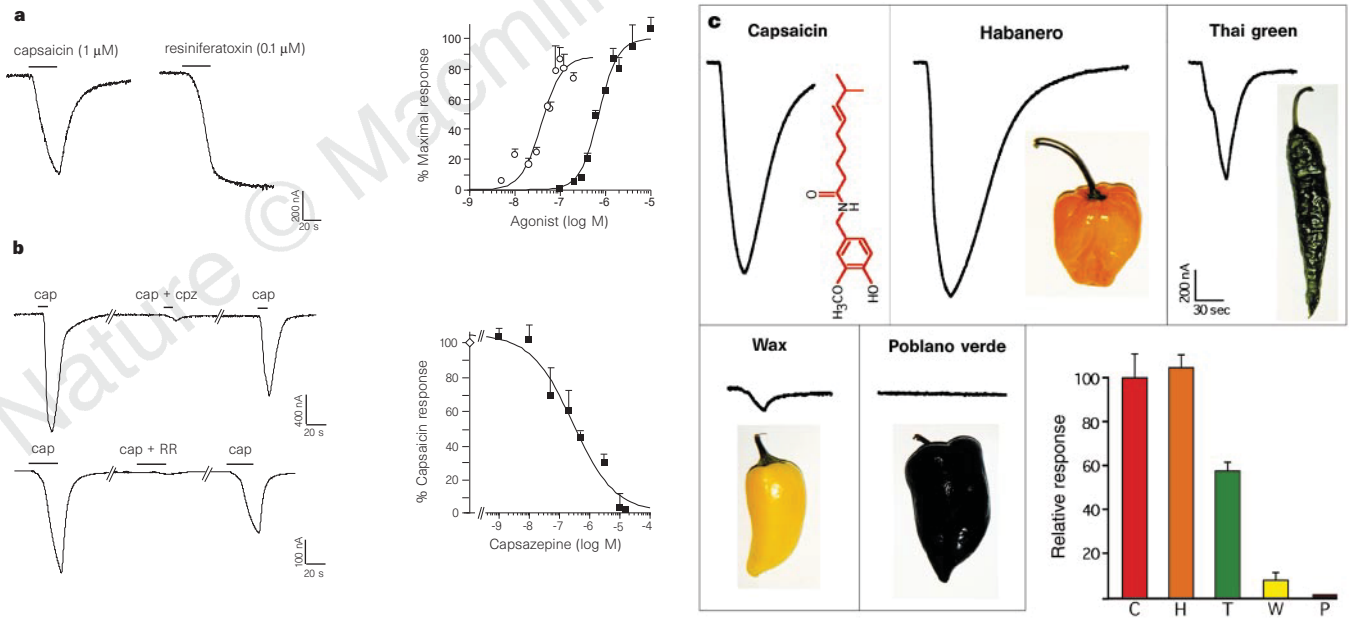


Figure 2 VR1 responds to purified vanilloids and pepper extracts. **a**, Activation of VR1 by capsaicin and resinferatoxin. Left, agonists were applied sequentially to the same *Xenopus* oocyte expressing VR1. Membrane currents were recorded in the whole-cell voltage-clamp configuration. Bars denote duration of agonist application. Right, concentration–response curve for capsaicin (filled squares) and resiniferatoxin (open circles). Membrane currents were normalized in each oocyte to a response obtained with 1 μM capsaicin and expressed as a percent of maximal response to capsaicin. Each point represents mean values (±s.e.m.) from five independent oocytes. The Hill equation was used to fit the response data. **b**, Antagonism by capsazepine (cpz) and ruthenium red (RR). Current tracing at top left shows reversible block of capsaicin (cap; 0.6 μM) response by capsazepine (cpz; 10 μM) after 2 min pretreatment. Slash marks represent washout periods of 2 and 3 min, respectively (n = 3). A capsazepine inhibition curve is shown to the right (n = 4 independent oocytes for each point). Current responses were normalized to that elicited by capsaicin alone in each oocyte. (0.6 μM, open diamond). Current tracing at bottom left shows reversible block of a capsaicin (0.6 μM)-evoked response by ruthenium red (RR; 10 μM). Slash marks denote washout periods of 2 and 12 min, respectively (n = 3). **c**, Responses to capsaicin (10 μM) and extracts derived from four varieties of peppers in oocytes expressing VR1 (30 s application). Bottom right, relative potencies of each pepper extract are plotted (mean ± s.e.m., n = 3). Values were normalized in each cell to responses obtained with capsaicin (10 μM). Extracts evoked no responses in water-injected cells. Reported pungencies for pepper varieties (in Scoville units) are: Habanero (H), 100,000–300,000; Thai green (T), 50,000–100,000; wax (W), 5,000–10,000; and Poblano verde (P), 1,000–1,500 (ref. 23). Capsaicin (C) is rated as 16×10^6 units.

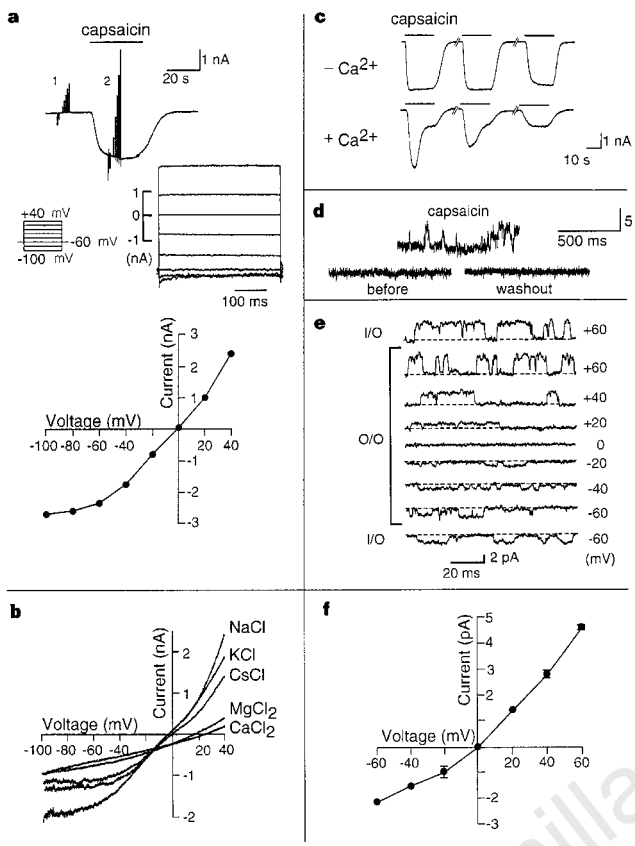


Figure 3 VR1 is a calcium-permeable, non-selective cation channel. Electrophysiological properties of capsaicin-activated currents in VR1-transfected mammalian HEK293 cells. **a**, VR1 currents are time independent, outwardly rectifying, and cation specific. A capsaicin-evoked inward current response (top) was analysed using a series of 400-ms step pulses (–100 to +40 mV; middle, left). Baseline currents (denoted as 1 on top trace) were subtracted from responses in the presence of agonist (2) to yield a series of agonist-evoked responses at different holding potentials (middle, right). These responses show outward rectification when plotted as a function of membrane voltage (bottom). Calcium-free standard bath solution and a caesium aspartate-filled recording electrode were used. **b**, Capsaicin elicits non-selective cation currents in VR1-transfected cells. Voltage ramps (–100 to +40 mV in 500 ms) were used to generate current–voltage curves in bath solutions with the indicated cationic compositions. Recording electrodes were filled with NaCl. Similar results were obtained with KCl- or CsCl-filled electrodes. Replacement of extracellular NaCl (140 mM) with equimolar KCl or CsCl did not significantly shift reversal potential (E_{rev} = -0.7 ± 1.2 mV, $n = 8$; -1.5 ± 1.0 mV, $n = 9$; -4.3 ± 0.9 mV, $n = 8$, respectively; $P_K/P_{Na} = 0.94$; $P_{Ca}/P_{Na} = 0.85$). Replacement of extracellular NaCl with isotonic (112 mM) $MgCl_2$ or $CaCl_2$ shifted E_{rev} to 14.4 ± 0.7 mV ($n = 5$) or 24.3 ± 2.3 mV ($n = 7$), respectively ($P_{Mg}/P_{Na} = 4.99$; $P_{Ca}/P_{Na} = 9.60$). **c**, Whole-cell current responses evoked by repeated capsaicin applications show desensitization in calcium-containing standard bath solution, but not in calcium-free solution. Capsaicin (1 μM) was applied every 5 min and CsCl was used as pipette solution. The ratios of current size at the end of the third application to the peak of the first application were $95.3 \pm 2.6\%$ ($n = 3$) in calcium-free solution, and $13.0 \pm 4.3\%$ ($n = 5$) in calcium-containing solution (t-test; $P < 0.00001$). **d**–**f**, Single-channel properties of capsaicin-evoked responses. Inside-out (I/O) or outside-out (O/O) patches were excised from VR1-transfected cells and analysed in symmetrical 140 mM NaCl. **d**, Traces obtained from a single O/O patch before, during and after capsaicin (1 μM) application to the bath solution ($V_{hold} = +40$ mV). Note multiple simultaneous channel openings in the presence of capsaicin. **e**, Traces obtained in the presence of capsaicin at the indicated holding potentials. Broken lines indicate the closed-channel level. No agonist-evoked channel activity was seen in cells transfected with vector alone ($n = 8$, not shown). **f**, Current–voltage curve of mean single-channel amplitudes (±s.e.m.) calculated from data shown in **e**, also exhibits pronounced outward rectification.

respectively). Hill coefficients derived from these analyses (1.95 and 2.08, respectively) suggest that full activation of the receptor involves the binding of more than one agonist molecule, again consistent with previously described properties of native vanilloid receptors[8,16]. Capsaicin-evoked responses in VR1-expressing oocytes were reversibly blocked by the competitive vanilloid receptor antagonist capsazepine at concentrations ($IC_{50} = 283.5$ nM) that inhibit native receptors[13] (Fig. 2b). Another pharmacological characteristic of vanilloid receptors, is their sensitivity to the non-competitive antagonist ruthenium red[17], which blocked capsaicin-evoked responses in a reversible manner (Fig. 2b). Responses to resiniferatoxin (50 nM) were also reversibly antagonized by capsazepine (5 μM) or ruthenium red (10 μM) (not shown).

As has been recognized for years, the relative pungencies of pepper varieties span an enormously wide range, reflecting, in part, differences in vanilloid content. Methods for rating peppers with respect to their relative 'hotness' have hitherto relied on subjective psychophysical assays[21] or on the biochemical determination of capsaicin content[22]. To further explore the connection between the biology and biochemistry of vanilloid action, we sought to determine whether the cloned vanilloid receptor could respond electrophysiologically to pepper extracts in proportion to their ability to evoke pain. Ethanol extracts were prepared from several capsicum varieties and their potencies relative to a saturating dose of capsaicin (10 μM) were determined in the oocyte expression system (Fig. 2c). Indeed, we found that the different 'hotness' of these pepper variants, as determined by subjective psychophysical ratings[23], correlated with their rank order potencies as activators of VR1.

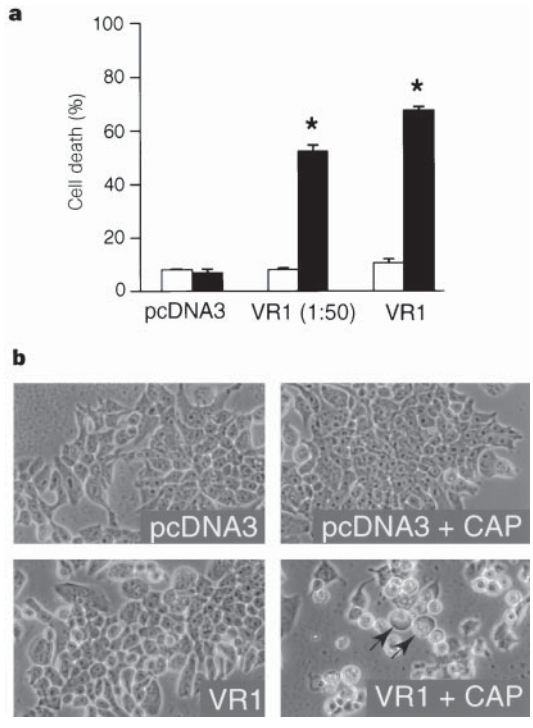


Figure 4 Capsaicin induces death of cells expressing the vanilloid receptor. **a**, HEK293 cells were transiently transfected with either vector alone (pCDNA3), VR1 cDNA diluted 1:50 in pCDNA3, or VR1 cDNA alone. After 7 h at 37 °C in the presence of capsaicin (3 μM, black bars) or vehicle (0.3% ethanol, white bars), the percentage of dead cells was determined. Data represent mean ± s.e.m. of triplicate determinations from a representative experiment. Asterisks indicate a significant difference from ethanol-treated cells (t-test, $P < 0.0001$). **b**, Phase-contrast photomicrographs of parallel cultures transfected with pCDNA3 or VR1 (1:50) before (left) or 4 h after (right, +CAP) addition of capsaicin (3 μM). Note the cytoplasmic swelling and eccentric position of cytoplasmic contents (arrows).

To explore the possibility that capsaicin mimics the action of a known chemical modulator of nociceptor function, we tested agents known to activate sensory neurons for their ability to evoke responses in HEK293 cells or oocytes expressing VR1. None of the agents tested gave positive responses, including adenosine triphosphate (50 μM), serotonin (10 μM), acetylcholine (300 μM), bradykinin (1 μM), substance P (10 μM), histamine (10 μM), glutamate (100 μM), and hypertonic saline (600 mOsm).

VR1 ion channel has high Ca^{2+} permeability

To characterize more fully the electrophysiological properties of the cloned receptor at both whole-cell and single-channel levels, we performed a series of patch-clamp studies on transfected mammalian cells expressing VR1. In the whole-cell configuration, VR1-transfected HEK293 cells showed robust inward current responses (at a holding potential of −60 mV) that developed with a short latency upon bath application of capsaicin (Fig. 3a). No such currents were observed in cells transfected with vector alone (not shown). In calcium-free medium, the capsaicin-evoked current did not vary with time, either at a constant holding potential of −60 mV or during voltage steps from −100 to +40 mV (in increments of 20 mV) (Fig. 3a). This property enabled us to characterize capsaicin-mediated currents under steady-state response conditions in subsequent experiments. Current–voltage relations derived from these data show that such responses exhibit prominent outward rectification resembling that observed in cultured dorsal root ganglion neurons[8] (Fig. 3a, bottom). Because the observed reversal potential was close to 0 mV ($E_{rev} = 0.5 \pm 0.9$ mV, $n = 13$), the capsaicin-mediated response must involve the opening of a cation-selective channel. In sensory neurons, vanilloid-evoked currents are carried by a mixture of monovalent and divalent cations[7–9], and we therefore conducted a series of ion substitution experiments to examine the relative contributions of various cations to capsaicin-evoked currents in VR1-expressing cells. Current–voltage relations established for cells bathed in solutions of differing cationic compositions show that VR1 does not discriminate among monovalent cations, but exhibits a notable preference for divalent cations (permeability sequence: $Ca^{2+} > Mg^{2+} > Na^{+} \approx K^{+} \approx Cs^{+}$) (Fig. 3b). The very high relative permeability of VR1 to calcium ions ($P_{Ca}/P_{Na} = 9.60$; $P_{Mg}/P_{Na} = 4.99$) exceeds that observed for most non-selective cation channels, and is similar to values reported for NMDA-type glutamate receptors and α7 nicotinic acetylcholine receptors ($P_{Ca}/P_{Na} = 10.6$ and 20, respectively)[24,25], both of which are noted for this property. With all bath solutions examined, an outwardly rectifying current–voltage relation was observed, although this feature was less prominent in bath solutions containing $MgCl_2$ or $CaCl_2$.

In cultured sensory neurons, electrophysiological analyses of vanilloid-evoked responses have shown them to be kinetically complex and to desensitize with continuous vanilloid exposure[20,26]. This electrophysiological desensitization (which might underlie aspects of physiological desensitization produced by vanilloids *in vivo*) seems to depend, in part, on the presence of extracellular calcium[26,27]. Indeed, in the absence of extracellular calcium, capsaicin-evoked responses in VR1-transfected cells showed little or no desensitization during prolonged agonist application or with successive agonist challenges (4.7 ± 2.3% decrease between first and third applications, $n = 3$ (Fig. 3c)). In contrast, responses evoked in calcium-containing bath solution consisted of at least two distinct components, one desensitizing (87 ± 4.3% decrease between first and third applications, $n = 5$) and one relatively non-desensitizing. Thus desensitization and multiphasic kinetics of vanilloid-evoked responses can be reproduced without a neuronal context and can be distinguished by their dependence on ambient calcium levels.

The behaviour of the VR1 response was also examined in membrane patches excised from transfected cells. In the presence

Figure 5 VR1 resembles store-operated channels. **a**, Predicted amino-acid sequence encoded by the vanilloid receptor cDNA VR1. Open boxes delineate ankyrin repeat domains, black boxes predicted transmembrane domains, the grey box a possible pore-loop, and filled circles predicted protein kinase A phosphorylation sites. **b**, Predicted membrane topology and domain structure of VR1. Outer (o) and inner (i) plasma membrane leaflets are indicated. **c**, Alignment of VR1 with related sequences. Identical residues are in black boxes and conservative substitutions are in grey. A partial sequence from a human EST is shown. Other sequences are from members of the putative store-operated calcium channel family. The Genbank accession number of the *Drosophila* TRP protein is p19334; others are indicated.

of capsaicin (but not its absence), large and well-resolved currents of unitary amplitude were observed ($n = 31$; Fig. 3d, e), indicating the existence of capsaicin-gated ion channels within these patches whose activation does not depend upon soluble cytoplasmic components. The current–voltage relation at the single-channel level was almost identical to that established in whole-cell configuration, owing to its outward rectification and reversal potential near 0 mV under similar ionic conditions (Fig. 3f). Unitary conductances of 76.7 pS at positive potentials and 35.4 pS at negative potentials were observed with sodium as the sole charge carrier. These single-channel properties are like those previously described for native vanilloid receptors[8,28]. It has been suggested that the site of vanilloid action may not be confined to the extracellular side of the plasma membrane, owing to the lipophilic nature of these compounds[6]. We found that capsaicin was able to produce identical responses when added to either side of a patch excised from a cell expressing VR1 (Fig. 3e), consistent with the notion that vanilloids can permeate or cross the lipid bilayer to mediate their effects. A less likely but formally consistent explanation is that vanilloid receptors have functionally equivalent capsaicin-binding sites on both sides of the plasma membrane.

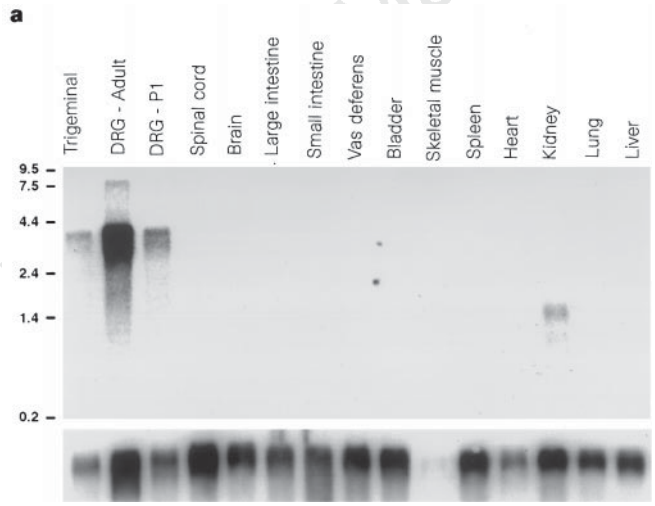
Capsaicin kills cells that express VR1

Capsaicin is an excitatory neurotoxin that selectively destroys primary afferent nociceptors *in vivo* and *in vitro*[5,9]. Is this selective toxicity solely a reflection of the specificity of vanilloid receptor expression, or does it depend on additional properties of sensory neurons or their environment? To address this question, we sought to determine whether capsaicin could kill non-neuronal cells that express vanilloid receptors *in vitro*. We found that, within several hours of continuous exposure to capsaicin, HEK293 cells transfected with VR1 died, as determined morphologically and by staining with vital dyes (Fig. 4). In contrast, cells transfected with vector alone were not killed by this treatment. The cell death was characterized by prominent cytoplasmic swelling, coalescence of cytoplasmic contents, and eventual lysis. Thus VR1 expression in a non-neuronal context can recapitulate the cytotoxicity observed in vanilloid-treated sensory neurons. Staining with Hoechst dye 33342 revealed no evidence of the nuclear fragmentation often associated with apoptotic cell death[29] (not shown). Together, these observations are consistent with necrotic cell death resulting from excessive ion influx, as has been proposed for vanilloid-induced death of nociceptors[7], glutamate-induced excitotoxicity[30], and neurodegeneration caused by constitutively activating mutations of various ion channels[31].

VR1 resembles TRP-related ion channels

The VR1 cDNA contains an open reading frame of 2,514 nucleotides that encodes a protein of 838 amino acids with a predicted relative molecular mass of 95,000 (M_r 95K) (Fig. 5a). Hydrophilicity analysis suggests that VR1 is a polytopic protein containing six transmembrane domains (predicted to be mostly β-sheet) with an additional short hydrophobic stretch between transmembrane regions 5 and 6 (Fig. 5b). The amino-terminal hydrophilic segment (432 amino acids) contains a relatively proline-rich region followed by three ankyrin repeat domains. The carboxy terminus (154 amino acids) contains no recognizable motifs.

A homology search of protein databases revealed significant similarities between VR1 and members of the family of putative store-operated calcium channels (SOCs), the prototypical members of which include the *Drosophila* retinal proteins TRP and TRPL[32,33] (Fig. 5c). Members of this family have been proposed to mediate the entry of extracellular calcium into cells in response to the depletion of intracellular calcium stores[34]. These proteins resemble VR1 with respect to their predicted topological organization and the presence of multiple N-terminal ankyrin repeats[33]. There is also striking



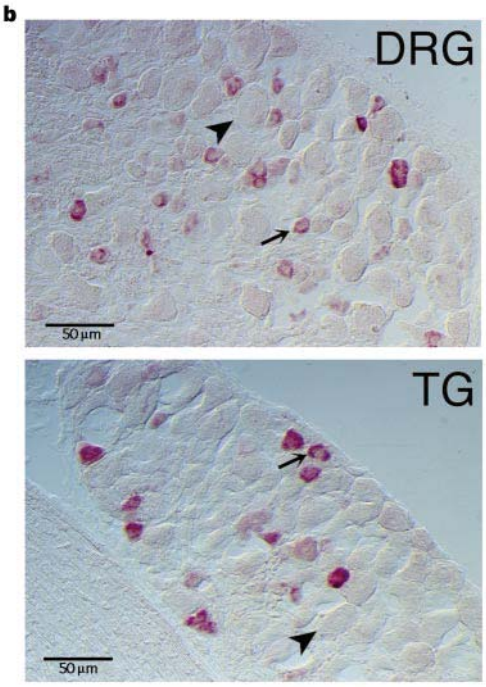


Figure 6 Vanilloid receptor expression is restricted to sensory neurons. **a**, Northern blot analysis shows that VR1 transcripts are confined to sensory ganglia. Poly(A)$^+$ RNAs were prepared from adult rats, except for the DRG-P1 sample, which was isolated from dorsal root ganglia of newborn pups. The blot was probed with ^{32}P-labelled VR1 cDNA, then re-probed with a ^{32}P-labelled cyclophilin cDNA to control for loading (bottom). Molecular size markers (kilobases) are shown on the left. **b**, *In situ* hybridization detects VR1 expression in a subset of sensory ganglion cells. Adult rat dorsal root ganglia (DRG) and trigeminal ganglia (TG) were probed with a digoxigenin-labelled, VR1-derived antisense riboprobe. Positive staining (purple) was confined to smaller-diameter cell bodies (arrows) and absent from large-diameter cell bodies (arrowheads). Control (sense) riboprobes did not stain these tissues (not shown).

amino-acid sequence similarity between VR1 and TRP-related proteins within and adjacent to the sixth transmembrane region, including the short hydrophobic region between transmembrane domains 5 and 6 that may contribute to the ion permeation path[33]. Outside these regions, VR1 shares little sequence similarity with TRP family members, suggesting that its evolutionary relationship to these proteins is distant. Given the high permeability of VR1 to calcium ions, we nonetheless considered the possibility that it might function as a SOC. To test this, we examined calcium-dependent inward currents in VR1-expressing oocytes whose intracellular calcium stores had been depleted by treatment with the compound thapsigargin. In water-injected control oocytes, a clear depletion-induced current was seen, as previously described[35] (not shown). In VR1-expressing oocytes, no quantitative or qualitative differences were observed in this response (not shown). Moreover, application of SKF 96365 (20 μM), an inhibitor of depletion-stimulated calcium entry[36], had no effect on capsaicin-evoked currents in VR1-expressing oocytes (not shown). Thus VR1 does not seem to be a functional SOC under these circumstances.

An expressed sequence tag (EST) database homology search revealed several human clones with a high degree of similarity to VR1 at both the nucleotide and predicted amino-acid levels. The similarity of one of these clones to the corresponding region of VR1 (Fig. 5c) is extremely high (68% amino-acid identity and 84% similarity within the region shown), suggesting that it is likely to be the human VR1 orthologue or a closely related subtype. Human EST clones corresponding to other domains of VR1 show comparable degrees of similarity (not shown), and could represent fragments of the same human transcript.

Sensory neuron-specific expression of VR1

The highly selective nature of capsaicin action suggests that vanilloid receptors serve as specific molecular markers for nociceptive neurons. Indeed, northern blot analysis showed that a mRNA species of approximately 4 kb is prominently and exclusively expressed in trigeminal and dorsal root sensory ganglia, both of which contain capsaicin-sensitive neurons (Fig. 6a). This transcript was absent from all other tissues examined, including spinal cord and brain. A much smaller RNA species (~1.5 kb) was detected in the kidney, but it is unclear whether this transcript could encode a functional VR1 protein. *In situ* hybridization histochemistry was used to assess the cellular pattern of VR1 expression within sensory ganglia (Fig. 6b). These experiments clearly show that within dorsal root and trigeminal ganglia, VR1 expression predominates in a subset of neurons with small diameters. This is in keeping with the observation that most capsaicin-sensitive neurons have cell bodies of relatively small to medium size[5,27]. In contrast to the prominent expression of VR1 transcripts in neurons of the dorsal root ganglion, no visible signal was observed in the adjacent spinal cord dorsal horn (not shown). Although binding sites for radiolabelled resiniferatoxin have been detected in the dorsal horn, they are believed to reside on presynaptic terminals that project from primary nociceptors with cell bodies located in the dorsal root ganglia[27]. Our results support this interpretation. Two other tissues that have been proposed to express capsaicin receptors are the nodose ganglion, which contains cell bodies of visceral nociceptors[27], and the preoptic area of the hypothalamus[2], which is involved in thermoregulation[37]. By using *in situ* hybridization methods, we did not detect VR1 expression at either location. Although these tissues might express VR1 at levels below the detection limit of our assay, vanilloid responsiveness here might be conferred by distinct VR subtypes. Indeed, VR heterogeneity has been proposed on the basis of biochemical studies[4,16].

Vanilloid receptor activated by noxious heat

The 'burning' quality of vanilloid-induced pain suggests that vanilloids and heat may evoke painful responses through a common molecular pathway. We therefore explored the effects of elevated temperature on VR1 activity. In initial studies, transfected HEK293 cells were subjected to fluorescent calcium imaging during

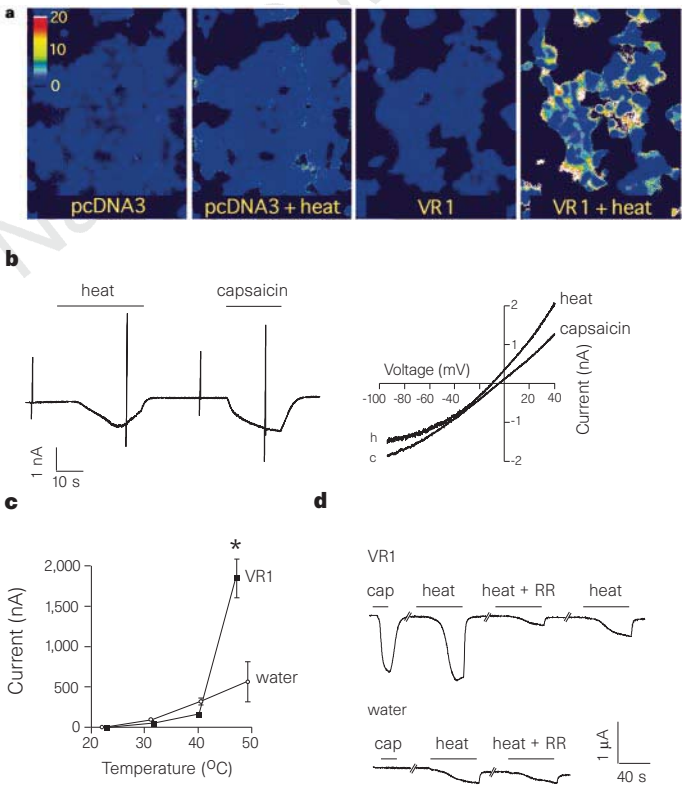


Figure 7 VR1 is activated by noxious thermal stimuli. **a**, HEK293 cells transiently transfected with VR1, but not vector alone (pcDNA3), exhibit a pronounced increase in cytoplasmic free calcium in response to heat. Cells were analysed before and immediately after addition of heated buffer (300 μl CIB at 65 °C was applied to cells in 150μl CIB at 22 °C). Under these conditions, cells were transiently exposed to a peak temperature of ~45 °C. Relative calcium concentrations are indicated by the colour bar, as in Fig. 1. **b**, Whole-cell patch-clamp analysis ($V_{hold} = -60$ mV) of VR1-transfected HEK293 cells reveals inward current responses to both heat and capsaicin. The temperature of the bath medium was raised from 22 to 48 °C (heat), and then restored to 22 °C, after which capsaicin (0.5 μM) was added to the bath (left trace). Ionic conditions were identical to those described in Fig. 3a. Voltage ramps (–100 to +40 mV in 500 ms) were applied before, between and during responses. Stimulus-induced current–voltage relations are shown on the right. **c**, VR1 expressed in *Xenopus* oocytes is activated by noxious but not innocuous heat. Oocytes injected with either VR1 cRNA or water were subjected to two-electrode voltage-clamp while the perfusate temperature was raised from 22.7 °C to the level indicated, then held constant for 60 s. The magnitudes of the resulting inward currents are shown as the mean ± s.e.m. (VR1, $n = 8$; water, $n = 6$ independent cells). The asterisk indicates a significant difference from water-injected oocytes (*t*-test, $P < 0.0005$). **d**, Ruthenium red (RR) inhibits heat-evoked responses in VR1-expressing oocytes. The current tracings shown were generated from representative VR1- or water-injected oocytes during successive applications of the indicated stimuli. VR1-injected oocytes exhibited the following mean inward current responses ±s.e.m. ($n = 5$): capsaicin (cap, 1 μM), 1,221 ± 148 nA; heat (50 °C), 2,009 ± 134 nA; heat plus RR (10 μM), 243 ± 47 nA. Inhibition by RR was significant (88 ± 2%, $n = 5$; paired *t*-test, $P < 0.0001$). In the absence of RR, no diminution in current was observed with successive heat pulses (not shown). Water-injected oocytes showed no response to capsaicin and much smaller responses to heat (338 ± 101 nA, $n = 5$). RR inhibited these responses by only 21 ± 26% ($n = 5$; paired *t*-test, $P < 0.1$).

a sudden increase in ambient temperature from 22 °C to ~45 °C. Under these conditions, cells transfected with vector alone exhibited only a mild, diffuse change in cytoplasmic free calcium (Fig. 7a, left). In contrast, a large proportion of cells expressing VR1 exhibited a pronounced increase in calcium levels within seconds of heat treatment (Fig. 7a, right). These responses subsided within a few minutes, and a subsequent challenge with capsaicin produced a characteristic calcium response (not shown), suggesting that the response to heat is a specific signalling event and not a consequence of nonspecific membrane perturbation or disruption of cell integrity. To determine whether specific heat-evoked membrane currents are associated with this phenomenon, VR1-expressing cells were examined using patch-clamp methods. Exposure of these cells to a rapid increase in temperature (22 °C to ~48 °C in 25 s, monitored using an in-bath thermistor) produced large inward currents (791 ± 235 pA at −60 mV, $n = 9$) that were typically similar in amplitude to that evoked by a subsequent application of capsaicin at 500 nM (Fig. 7b). Both heat- and vanilloid-evoked responses showed outward rectification, suggesting that they are mediated by the same entity (Fig. 7b). By comparison, thermally evoked responses of control, vector-transfected cells were much smaller (131 ± 23 pA, $n = 8$) and exhibited no rectification (not shown). The heat response in VR1-transfected cells desensitized during stimulus application, whereas that observed in vector-transfected cells did not. These results suggest that VR1 is acting as a thermal transducer, either by itself or in conjunction with other cellular components.

To determine whether VR1 could mediate similar responses to heat in a different cellular environment, we extended these studies to the oocyte system. In control, water-injected oocytes, acute elevation of perfusate temperature produced a small inward current that increased linearly up to 50 °C (Fig. 7c). VR1-expressing oocytes exhibited similar responses at temperatures up to 40 °C, but above this threshold their responses were significantly larger than those of controls. Thus, even in this non-mammalian context, VR1 expression confers heat sensitivity with a temperature–response profile that is remarkably consistent with that reported for thermal nociceptors[1]. Pharmacological experiments also suggest that VR1 is involved directly in this thermal response: application of ruthenium red reduced significantly (88 ± 2%, $n = 5$) the response of VR1-expressing oocytes to heat, whereas the smaller response seen in control cells was reduced by only 21 ± 26% ($n = 5$; Fig. 7d). Taken together, these observations strongly support the hypothesis that VR1 is activated by noxious, but not innocuous, heat.

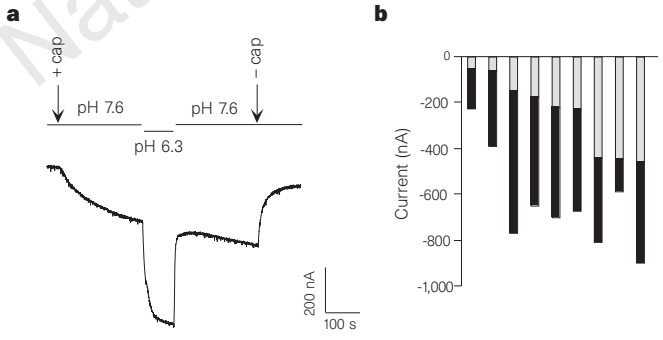


Figure 8 Hydrogen ions potentiate the effect of capsaicin on VR1. **a**, Augmentation of capsaicin-evoked current response by reduced pH in VR1-expressing oocytes. Capsaicin (cap, 0.3 μM) was administered throughout the time period spanned by the arrows. The pH of the bath solution was changed during the experiment as indicated by the horizontal bars. VR1-expressing oocytes exhibited no responses to pH 6.3 bath solution without capsaicin; water-injected control oocytes exhibited no responses to either capsaicin or pH 6.3 bath solution (not shown). **b**, Summary of current responses obtained from 9 independent VR1-expressing oocytes. The grey portion of each bar indicates peak current evoked by capsaicin at pH 7.6; the black portion represents the additional current evoked by changing the pH to 6.3.

Protons may be endogenous modulators of VR1

A reduction in tissue pH resulting from infection, inflammation or ischaemia can produce pain in mammals. It has therefore been proposed that protons might act as endogenous activators or modulators of vanilloid receptors[38–40]. To address this possibility, we examined the effects of hydrogen ions on the cloned vanilloid receptor using the oocyte expression system. We investigated whether an abrupt reduction in bath solution pH, from 7.6 to 5.5, was sufficient to activate VR1 in the absence of capsaicin. Fewer than 10% of VR1-expressing oocytes treated in this way exhibited a large inward current (not shown), suggesting that hydrogen ions alone cannot efficiently activate this protein. We next assessed the effect of reduced pH on the responsiveness of VR1 to capsaicin. VR1-expressing oocytes were treated with a submaximal concentration of capsaicin (300 nM) at pH 7.6 (Fig. 8). Once their current responses reached a relatively stable plateau, the oocytes were exposed to a solution containing the same concentration of capsaicin at pH 6.3. Under these conditions, the inward current rapidly increased to a new plateau up to fivefold greater in magnitude than the first. Upon returning to pH 7.6, the oocyte response subsided to its initial plateau, and upon the removal of capsaicin it returned to baseline. This potentiation was seen only with subsaturating concentrations of agonist, as reduced pH did not augment responses to 10 μM capsaicin (not shown). These results suggest that, although hydrogen ions alone are not sufficient to activate VR1, they can markedly potentiate capsaicin-evoked responses, presumably by increasing capsaicin potency.

Discussion

Opioids and vanilloids are natural products whose physiological effects are apparently so seductive or desirable that their use has permeated diverse cultures for thousands of years. Consequently, it has long been assumed that their molecular modes of action must involve important physiological processes that underlie endogenous mechanisms of pain sensation and regulation. In the study of opioid action, this assumption led to the discovery of the principal signalling system that suppresses pain *in vivo*[41]. We expected that the identification of the biological target of vanilloid action would similarly illuminate a fundamental mechanism of pain production. By pursuing this hypothesis, we have defined a molecular component of the nociceptive pathway that transduces two of the principal types of painful stimuli: thermal and chemical.

The cloning of VR1 demonstrates unequivocally that the molecular target of capsaicin action on sensory neurons is a proteinacious ion channel. VR1 is structurally related to the TRP family of ion channels that have been proposed to mediate the influx of extracellular calcium in response to depletion of intracellular calcium stores. The precise physiological roles and mechanisms of activation of these channels have been the subject of much debate[34]. It has been proposed that these channels are gated by diffusible small molecules released from depleted intracellular calcium stores, or alternatively that gating involves direct allosteric interactions with store-associated proteins. If TRP-related channels are gated by diffusible molecules, these molecules might bind to a site analogous to that used by vanilloid compounds to activate VR1. Indeed, SKF 96365, an inhibitor of depletion-mediated calcium entry, contains two methoxyphenyl moieties[36], which resemble vanillyl groups. An understanding of VR1 activation may therefore provide new molecular insights into these broader biochemical issues. In any case, the distant but clear molecular relationship between VR1 and TRP family members makes it likely that this group of ion channels subserves diverse physiological functions.

The cloned capsaicin receptor is activated not only by vanilloid compounds but also by thermal stimuli within the noxious temperature range. The temperature–response profile of VR1 matches very closely those reported for heat-evoked pain responses in humans and animals, and for heat-evoked currents in cultured sensory neurons[1,42,43]. Several other properties of VR1-mediated heat responses are like those seen in whole animals or in cultured sensory neurons. For instance, ruthenium red, which blocks VR1 activity in mammalian cells or oocytes, also blocks heat-evoked nociceptive responses in the rabbit ear[44]. In addition, the heat-evoked currents observed in both VR1-expressing cells and cultured sensory neurons are carried through outwardly rectifying, non-selective cation channels[42,43]. Electrophysiological recordings from cultured sensory neurons reveal a striking concordance between responsiveness to capsaicin and heat[45]. Our findings, together with these observations, suggest that an *in vivo* role of vanilloid receptors is to detect noxious heat. But not all characteristics of endogenous heat responses resemble those mediated by VR1. In the whole animal, capsaicin pretreatment reduces responsiveness to noxious thermal stimuli in some, but not all, physiological contexts[2,3,5]. Similarly, in cultured sensory neurons, some heat-activated currents are reported to be insensitive to ruthenium red and to exhibit a lower relative contribution from calcium ions[42,43]. Thus responses to noxious thermal stimuli may be transduced through multiple molecular pathways, only some of which may involve VR1. It is presently unclear whether thermosensitivity is an intrinsic physical property of VR1, but the fact that VR1 confers heat responsiveness to both mammalian cells and frog oocytes indicates that any other requisite component(s) must be widely expressed. Disruption of the VR1 gene in mice should help to clarify these issues.

The activation of VR1 by heat does not exclude the possibility that small molecules or other endogenous factors also modulate vanilloid-receptor function. It has been proposed, for instance, that protons produce pain by activating vanilloid receptors[38]. Although we did not observe consistent activation of VR1 by protons alone, we did find that protons can potentiate vanilloid-evoked responses in VR1-expressing oocytes. This is consistent with the observation that hydrogen ions potentiate the effects of low concentrations of capsaicin on cultured sensory neurons[39,40]. Moreover, in preliminary experiments, we have observed that low pH can also increase the response of VR1 to noxious thermal stimuli in the oocyte expression system. Thus the increased response to noxious stimuli (hyperalgesia) that accompanies inflammation and ischaemia might result, in part, from an enhancement of vanilloid receptor function by the excess hydrogen ions they produce[38]. If this is the case, VR1 could provide an important model system for the *in vitro* study of hyperalgesia.

The multiple consequences of vanilloid receptor activation make it possible that this protein is involved in diverse human disease states ranging from congenital pain insensitivity to chronic pain syndromes. The cloning of VR1 provides both a molecular probe with which to address these possibilities and a defined target for the development of new analgesic agents. □

Methods

Expression cloning and DNA analysis. A rodent dorsal root ganglion plasmid cDNA library was constructed in pCDNA3 (Invitrogen) essentially as described[46], using a mixture of polyadenylated RNA from newborn rat and adult mouse dorsal root ganglia to generate first-strand cDNA. The resulting 2.4×10^6 independent bacterial clones were divided into 144 pools. HEK293 cells expressing the SV40 large T antigen (gift from T. Livelli) and maintained in DMEM (supplemented with 10% fetal bovine serum (Hyclone), penicillin, streptomycin and L-glutamine) were transfected with plasmid DNA from individual pools using a calcium phosphate precipitation kit (Specialty Media). The next day the cells were replated onto eight-well polyornithine-coated chambered coverglasses (Applied Scientific). Between 6 and 24 h later they were loaded with Fura-2 (30 min at 37 °C) in CIB buffer containing (in mM) 130 NaCl, 3 KCl, 2.5 $CaCl_2$, 0.6 $MgCl_2$, 1.2 $NaHCO_3$, 10 glucose, 10 HEPES, pH 7.45, with 10 μM Fura-2 acetoxymethyl ester and 0.02% pleuronic acid (Molecular Probes), then rinsed twice with CIB. Ratiometric calcium imaging was performed using a Nikon Diaphot fluorescence microscope equipped with a variable filter wheel (Sutter Instruments) and an intensified CCD camera (Hamamatsu). Dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolour ratiometric images monitored during the experiment (Metafluor software, Universal Imaging). Cells were initially imaged in 200 μl CIB, after which 200 μl CIB containing capsaicin at twice the desired concentration was added. After stimulation, cells were observed for 60–120 s. For each library pool, one microscopic field (300–500 cells) was assayed in each of eight wells. DNA sequencing was performed using an automated sequencer (ABI). Homology searches were performed against the non-redundant Genbank database and against an EST database (dbEST). Hydrophilicity was calculated using the Hopp–Woods algorithm[47]. VR1 was determined to be of rat origin by sequencing an independent cDNA isolated from a rat dorsal root ganglia library and a polymerase chain reaction (PCR) product derived from mouse dorsal root ganglia.

Oocyte electrophysiology. cRNA transcripts were synthesized from *Not*1-linearized VR1 cDNA templates using T7 RNA polymerase[46]. Defolliculated *Xenopus laevis* oocytes were injected with 0.5–5 ng VR1 cRNA. Four to seven days after injection, two-electrode voltage-clamp recording was performed ($E_{hold} = -60$ mV for IC_{50} curve and thermal stimulation experiments, and –40 mV for all other experiments) using a Geneclamp 500 amplifier (Axon Instruments) and a MacLab A/D converter (Maclab). The recording chamber was perfused at a rate of 2 ml min^{-1} with frog Ringer's solution containing (in mM) 90 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 0.1 $BaCl_2$, 1.0 $MgCl_2$ and 10 HEPES, pH 7.6, at room temperature. $CaCl_2$ (2 mM) was used instead of $BaCl_2$ when generating the capsazepine inhibition curve. Thermal stimuli were applied using a preheated bath solution and temperature was monitored using a thermistor placed next to the oocyte. For store-operated current assays, oocytes were incubated for 1–2 h in calcium-free, barium-free frog Ringer's solution containing 1 mM EGTA and 1 μM thampsigargin. During voltage-clamp recording, these oocytes were intermittently exposed to frog Ringer's solution containing 2 mM Ca^{2+} and no EGTA to detect calcium-dependent currents (15-s pulses at 2-min intervals)[35]. Capsazepine, ruthenium red and thapsigargin were purchased from RBI, SKF 96365 from ICN, and all other chemicals from Sigma. Finely chopped whole peppers (15 g) were extracted overnight at room temperature with 50 ml absolute ethanol. Soluble extracts were concentrated 15-fold by vacuum desiccation, then diluted 1,000-fold in frog Ringer's solution for electrophysiological assay.

Mammalian cell electrophysiology. Patch-clamp recordings were performed with transiently transfected HEK293 cells at 22 °C. Standard bath solution for whole-cell recordings contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH). In calcium-free bath solution, $CaCl_2$ was replaced with 5 mM EGTA. For monovalent cation substitution experiments, after the whole-cell configuration was obtained in standard both solution, the bath solution was changed to (in mM) 140 NaCl (or KCl or CsCl), 10 glucose and 10 HEPES (adjusted to pH 7.4 with NaOH, KOH or CsOH, respectively) and the reversal potential measured using voltage ramps (see Fig. 3 legend). For divalent cation permeability experiments, the bath solution was changed to (in mM) 110 $MgCl_2$ (or $CaCl_2$), 2 $Mg(OH)_2$ (or $Ca(OH)_2$), 10 glucose, 10 HEPES, pH 7.4 (adjusted with HCl). Bath solution for outside-out patch recordings and pipette solution for inside-out patch recordings contained (in mM) 140 NaCl, 10 HEPES, pH 7.4 (adjusted with NaOH). Bath solution for inside-out patch recordings and pipette solutions for outside-out patch recordings and ion substitution experiments contained (in mM) 140 NaCl, 10 HEPES, 5 EGTA, pH 7.4 (adjusted with NaOH). Pipette solution for other whole-cell recordings contained (in mM) 140 CsCl (or 130 CsAspartate and 10 NaCl), 5 EGTA, 10 HEPES, pH 7.4 (adjusted with CsOH). Liquid junction potentials were measured directly in separate experiments; they did not exceed 3 mV with solutions used and no correction for this offset was made. Whole-cell recording data were sampled at 20 kHz and filtered at 5 kHz for analysis (Axopatch 200 amplifier with pCLAMP software, Axon Instruments). Single-channel recording data were sampled at 10 kHz and filtered at 1 kHz. Permeability ratios for monovalent cations to Na (P_X/P_{Na}) were calculated as follows: $P_X/P_{Na} = \exp(\Delta V_{rev} F/RT)$, where V_{rev} is the reversal

potential, F is Faraday's constant, R is the universal gas constant, and T is absolute temperature. For measurements of divalent permeability, P_Y/P_{Na} was calculated as follows: $P_Y/P_{Na} = [Na^+]_i \exp(\Delta V_{rev}F/RT)(1 + \exp(\Delta V_{rev}F/RT))/4[Y^{2+}]_O$ (ref. 48), where the bracketed terms are activities. Assumed ion activity coefficients are 0.75 for monovalents and 0.25 for divalents[48].

Cell death, northern blot, and *in situ* hybridization analyses. For cell death assays, HEK293 cells were transfected as described above. Cells were rinsed twice with PBS 14 h later and re-fed with medium containing either capsaicin (3 μM) or vehicle alone (ethanol, 0.3% final). After 7 h, cells were collected and the number of dead cells quantified with an ethidium homodimer and a calcein green-containing cell viability kit (Molecular Probes). For northern analysis, rat-derived poly(A)$^+$ RNA was purified as described[49] or with the FastTrack kit (Invitrogen). Approximately 2 μg of each sample was electrophoresed through a 0.8% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham), and hybridized at high stringency with a ^{32}P-labelled probe representing the entire VR1 cDNA. For *in situ* hybridization histochemistry, adult female Sprague-Dawley rats were anaesthetized and perfused with 4% paraformaldehyde in PBS. Tissues were dissected, frozen in liquid N_2 and embedded in OCT mounting medium. Cryostat sections (15 μm thick) were processed and probed with a digoxigenin-labelled cRNA generated by *in vitro* transcription of a 1-kb fragment of the VR1 cDNA (nucleotides 1513–2482) (Genius kit, Boehringer Mannheim). Sections were developed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments according to the manufacturer's instructions.

Received 20 August; accepted 24 September 1997.

1. Fields, H. L. *Pain* (McGraw-Hill, New York, 1987).
2. Szolcsanyi, J. in *Capsaicin in the Study of Pain* (ed. Wood, J.) 1–26 (Academic, London, 1993).
3. Campbell, E. in *Capsaicin and the Study of Pain* (ed. Wood, J.) 255–272 (Academic, London, 1993).
4. Szallasi, A. & Blumberg, P. M. Vanilloid receptors: new insights enhance potential as a therapeutic target. *Pain* **68,** 195–208 (1996).
5. Jancso, G., Kiraly, E. & Jancso-Gabor, A. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurons. *Nature* **270,** 741–743 (1977).
6. James, I. F., Ninkina, N. N. & Wood, J. N. in *Capsaicin in the Study of Pain* (ed. Wood, J. N.) 83–104 (Academic, London, 1993).
7. Bevan, S. & Szolcsanyi, J. Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol. Sci.* **11,** 330–333 (1990).
8. Oh, U., Hwang, S. W. & Kim, D. Capsaicin activates a nonselective cation channel in cultured neonatal rat dorsal root ganglion neurons. *J. Neurosci.* **16,** 1659–1667 (1996).
9. Wood, J. N. *et al.* Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.* **8,** 3208–3220 (1988).
10. Feigin, A. M., Aaronov, E. V., Bryant, B. P., Teeter, J. H. & Brand, J. G. Capsaicin and its analogs induce ion channels in planar lipid bilayers. *Neuroreport* **6,** 2134–2136 (1995).
11. Szolcsanyi, J. & Jancso-Gabor, A. Sensor effects of capsaicin congeners I. Relationship between chemical structure and pain-producing potency of pungent agents. *Drug Res.* **25,** 1877–1881 (1975).
12. Szolcsanyi, J. & Jancso-Gabor, A. Sensory effects of capsaicin congeners II. Importance of chemical structure and pungency in desensitizing activity of capsaicin-like compounds. *Drug Res.* **26,** 33–37 (1976).
13. Bevan, S. *et al.* Capsazepine: a competitive antagonist of the sensory neuron excitant capsaicin. *Br. J. Pharmacol.* **107,** 544–552 (1992).
14. deVries, D. J. & Blumberg, P. M. Thermoregulatory effects of resiniferatoxin in the mouse: comparison with capsaicin. *Life Sci.* **44,** 711–715 (1989).
15. Szallasi, A. & Blumberg, P. M. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience* **30,** 515–520 (1989).
16. Szallasi, A. The vanilloid (capsaicin) receptor: Receptor types and species specificity. *Gen. Pharmacol.* **25,** 223–243 (1994).
17. Dray, A., Forbes, C. A. & Burgess, G. M. Ruthenium red blocks the capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory neurones as well as the activation of peripheral nociceptors in vitro. *Neurosci. Lett.* **110,** 52–59 (1990).
18. Tsien, R. Y. Fluorescent probes of cell signaling. *Annu. Rev. Neurosci.* **12,** 227–253 (1989).
19. Winter, J., Dray, A., Wood, J. N., Yeats, J. C. & Bevan, S. Cellular mechanism of action of resiniferatoxin: a potent sensory neuron excitotoxin. *Brain Res.* **520,** 131–140 (1990).
20. Liu, L. & Simon, S. A. A rapid capsaicin-activated current in rat trigeminal ganglion neurons. *Proc. Natl Acad. Sci. USA* **91,** 738–741 (1994).
21. Scoville, W. Note on capsicums. *J. Am. Pharm. Assoc.* **1,** 453–454 (1912).
22. Woodbury, J. E. Determination of capsicum pungency by high pressure liquid chromatography and spectrofluorometric determination. *J. Assoc. Official Anal. Chem.* **63,** 556–558 (1980).
23. Berkley, R. & Jacobson, E. *Peppers: A Cookbook* (Simon and Schuster, New York, 1992).
24. Mayer, M. L. & Westbrook, G. L. Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. *J. Physiol. (Lond.)* **394,** 501–527 (1987).
25. Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J. A. & Patrick J. W. Molecular cloning, functional properties, and distribution of rat brain a7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* **13,** 596–604 (1993).
26. Yeats, J. C., Docherty, R. J. & Bevan, S. Calcium-dependent and -independent desensitization of capsaicin-evoked responses in voltage-clamped adult rat dorsal root ganglion (DRG) neurones in culture. *J. Physiol. (Lond.)* **446,** 390*P* (1992).
27. Holzer, P. Capsaicin: Cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.* **43,** 143–201 (1991).
28. Forbes, C. A. & Bevan, S. Single channels activated by capsaicin in patches of membrane from adult rat sensory neurones in culture. *Neurosci. Lett.* (suppl.) **32,** S3 (1988).
29. Crem, R. J., Fechheimer, M. & MIller, L. K. Prevention of apoptosis by a Bacculovirus gene during infection of insect cells. *Science* **254,** 1388–1390 (1991).
30. Choi, D. W. Glutamate receptors and the induction of excitotoxic neuronal death. *Prog. Brain Res.* **100,** 47–51 (1994).
31. Hong, K. & Driscoll, M. A transmembrane domain of the putative channel subunit MEC-4 influences mechanotransduction and neurodegeneration in *C. elegans*. *Nature* **367,** 470–473 (1994).
32. Montell, C. & Rubin, G. M. Molecular characterization of the *Drosophila trp* locus: A putative integral membrane protein required for phototransduction. *Neuron* **2,** 1313–1323 (1989).
33. Hardie, R. C. & Minke, B. Novel Ca^{2+} channels underlying transduction in *Drosophila* photoreceptors: implications for phosphoinositide-mediated Ca^{2+} mobilization. *Trends Neurosci.* **16,** 371–376 (1993).
34. Clapham, D. E. TRP is cracked, but is CRAC TRP? *Neuron* **16,** 1069–1072 (1996).
35. Petersen, C. C. H., Berridge, M. J., Borgese, M. F. & Bennett, D. L. Putative capacitative calcium entry channels: expression of *Drosophila* trp and evidence for the existence of vertebrate homologs. *Biochem. J.* **311,** 41–44 (1995).
36. Merritt, J. E. *et al.* SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.* **271,** 515–522 (1990).
37. Satinoff, E. Behavioral thermoregulation in response to local cooling of the rat brain. *Am. J. Physiol.* **206,** 1389–1394 (1964).
38. Bevan, S. & Geppetti, P. Protons: small stimulants of capsaicin-sensitive sensory nerves. *Trends Neurosci.* **17,** 509–512 (1994).
39. Petersen, M. & LaMotte, R. H. Effect of protons on the inward current evoked by capsaicin in isolated dorsal root ganglion cells. *Pain* **54,** 37–42 (1993).
40. Kress, M., Fetzer, S., Reeh, P. W. & Vyklicky, L. Low pH facilitates capsaicin responses in isolated sensory neurons of the rat. *Neurosci. Lett.* **211,** 5–8 (1996).
41. Snyder, S. H. Opiate receptors and internal opiates. *Sci. Am.* **236,** 44–56 (1977).
42. Cesare, P. & McNaughton, P. A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. *Proc. Natl Acad. Sci. USA* **93,** 15435–15439 (1996).
43. Reichling, D. B. & Levine, J. D. Heat transduction in rat sensory neurons by calcium-dependent activation of a cation channel. *Proc. Natl Acad. Sci. USA* **94,** 7006–7011 (1997).
44. Amann, R., Donnerer, J. & Lembeck, F. Activation of primary afferent neurons by thermal stimulation: influence of Ruthenium Red. *Naunyn Schmiedeberg's Arch. Pharmacol.* **341,** 108–113 (1990).
45. Kirschstein, T., Busselberg, D. & Treede, R. D. Coexpression of heat-evoked and capsaicin-evoked inward currents in acutely dissociated rat dorsal root ganglion neurons. *Neurosci. Lett.* **231,** 33–36 (1997).
46. Brake, A., Wagenbach, M. J. & Julius, D. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371,** 519–523 (1994).
47. Hopp, T. P. & Woods, K. R. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl Acad. Sci. USA* **78,** 3824–3828 (1981).
48. Valera, S. *et al.* A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature* **371,** 516–519 (1994).
49. Cathala, G. *et al.* Laboratory methods: A method for isolation of intact, translationally active ribonucleic acid. *DNA* **2,** 329–335 (1983).

Acknowledgements. We thank H. Kong, M. Chao and A. Brake for the dorsal root ganglian cDNA and plasmid DNA used in library construction; T. Livelli for HEK293 cells and advice regarding transfection; J. Trafton for guidance with calcium imaging proceudres; N. Guy for tissue sections; J. Poblete for technical assistance; A. Basbaum and M. Dallman for comments on the manuscript; and A. Brake and H. Ingraham for advice and encouragement. M.J.C. is a recipient of an American Cancer Society postdoctoral fellowship and a NARSAD young investigator award. This work was supported by grants from the NIH.

Correspondence and requests for materials should be made to D.J. (e-mail: julius@socrates.ucsf.edu). The Genbank accession number for VR1 is AF029310.

Published in final edited form as:
J Neurosci. 2013 March 27; 33(13): 5533–5541. doi:10.1523/JNEUROSCI.5788-12.2013.

The cellular code for mammalian thermosensation

Leah A. Pogorzala, **Santosh K. Mishra**, and **Mark A. Hoon***
Molecular Genetics Unit, Laboratory of Sensory Biology, NIDCR, NIH, Building 49, Room 1A16, 49 Convent Drive, Bethesda MD20892

Abstract

Mammalian somatosenory neurons respond to thermal stimuli allowing animals to reliably discriminate hot from cold and select their preferred environments. We previously generated mice that are completely insensitive to temperatures from noxious cold to painful heat (−5 to 55 °C) by ablating several different classes of nociceptor early in development. Here we have adopted a selective ablation strategy in adult mice to dissect this phenotype and thereby demonstrated that separate populations of molecularly defined neurons respond to hot and cold. TRPV1-expressing neurons are responsible for all behavioral responses to temperatures between 40 and 50°C, while TRPM8-neurons are required for cold aversion. We also show that more extreme cold and heat activate additional populations of nociceptors including cells expressing Mrgprd. Thus, although eliminating Mrgprd-neurons alone does not affect behavioral responses to temperature, when combined with ablation of TRPV1- or TRPM8-cells, it significantly decreases responses to extreme heat and cold respectively. Notably, ablation of TRPM8-neurons distorts responses to preferred temperatures suggesting that the pleasant thermal sensation of warmth may in fact just reflect reduced aversive-input form TRPM8 and TRPV1-neurons. As predicted by this hypothesis, mice lacking both these classes of thermosensor exhibited neither aversive nor attractive responses to temperatures between 10 and 50 °C. Taken together these results provide a simple cellular basis for mammalian thermosensation whereby two molecularly defined classes of sensory neurons detect and encode both attractive and aversive cues.

Keywords

TRPV1; TRPM8; Mrgprd; hot; cold; pain; temperature

Introduction

Thermosensation provides valuable information about the environment and triggers strong emotional and behavioral responses over a wide temperature range. For example, noxious heat and cold are highly unpleasant sensations that trigger powerful escape reactions in most animals. However, mammalian thermosensation does far more than simply trigger reflex withdrawal from painful heat or cold: for instance, humans reliably distinguish unpleasantly hot and cold stimuli, while intermediate temperatures (e.g. cool or warm) can be very pleasant and drive attractive rather than aversive responses.

The ability of mammals to sense the temperature in their environment relies primarily on specialized somatosensory neurons in the trigeminal and dorsal root ganglia (DRG) that

*Correspondence to: Mark Hoon, mark.hoon@nih.gov, 301-402-6613.

Author Contributions: We are grateful to Nick Ryba for encouragement and helpful advice and Drs. Minh Nguyen and Lars von Buchholtz as well as members of MGU for valuable suggestions. Transgenic mice were generated by Andrew Cho in the NIDCR-core. We are very grateful to Dr. David Anderson for providing Mrgprd-DTR mice. This research was supported by the intramural research program of the NIH, NIDCR (MAH).

project axonal processes to the outer layers of the skin (Woolf and Ma, 2007). How are thermal cues encoded in these neurons at the periphery?

A major advance in the understanding of mammalian temperature sensation came with the characterization of TRPV1 as a high temperature and capsaicin gated-channel that is expressed in heat sensitive DRG neurons (Caterina et al., 1997). Subsequent identification of TRPM8 as a low temperature sensor (McKemy et al., 2002, Peier et al., 2002) as well as several other TRP-channels that appear to be stimulated by either heating or cooling led to the proposal that multiple TRP-channels act in concert as differentially tuned "molecular thermometers" to orchestrate appropriate responses over a wide range of temperatures (Jordt et al., 2003, Patapoutian et al., 2003).

In keeping with its role as a putative cold temperature sensor, knockout of TRPM8 results in a large selective deficit in an animal's ability to detect cool (15 – 25 °C) temperatures (Bautista et al., 2007, Colburn et al., 2007, Dhaka et al., 2007). However, knockout of other thermosensory TRP-channels, provides more modest support for this model (Kwan et al., 2006, Vriens et al., 2011) and the limited thermosensitive defect of TRPV1-KO mice has led to questions about the role of TRPV1 in vivo (Woodbury et al., 2004, Park et al., 2011). Nonetheless, agonist dependent ablation (Karai et al., 2004, Mishra and Hoon, 2010) or silencing (Cavanaugh et al., 2009) of TRPV1-neurons, resulted in significant deficits in sensing hot, suggesting that TRPV1 marks heat sensitive neurons. Unfortunately, resiniferatoxin-mediated ablation of TRPV1-cells is never complete (Mishra and Hoon, 2010) and the capsaicin induced lesions (Cavanaugh et al., 2009) are largely undefined reducing the strength of conclusions that can be drawn from this type of approach.

Recently, we generated mice that were completely insensitive to a very broad range of thermal stimuli (from −5 °C to 55 °C) (Mishra et al., 2011). In these animals, TRPV1 mediated expression of diphtheria toxin (DTA) resulted in ablation of several different molecular classes of nociceptor including the TRPV1-, TRPM8- and Mrgpr-neurons because TRPV1-DTA is expressed in common precursor cells (Mishra et al., 2011). Here we used a different molecular genetic approach to selectively eliminate each of these neural classes in adult mice and thereby delineated a simple logic for detection of temperature spanning the entire physiologically relevant range.

Materials and Methods

Animal models

All procedures followed the NIH Guidelines for the care and use of laboratory animals, and were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. TRPV1-DTR and TRPM8-DTR mice are BAC transgenics; BACs were engineered by recombineering (Lee et al., 2001) using RP23-181P10 and RP24-78N24 respectively to generate transgenic constructs (Figure 1a). As expected before DT treatment transgenically expressed GFP was readily detected in the appropriate number of somatosensory neurons. However, although we searched extensively outside peripheral ganglia in the brain and other organs that have been reported to express TRPV1 (Cavanaugh et al., 2011) we found no expression of GFP even using sensitive antibody staining techniques. Thus these BAC-transgenes selectively target DTR to the appropriate class of somatosensory neuron (see Figure 1 for additional characterization). The other strains have been described previously: Mrgprd-DTR (Cavanaugh et al., 2009) (a generous gift from David Anderson), TRPV1-DTA (Mishra et al., 2011), TRPM8 $^{-/-}$ (Bautista et al., 2007) and TRPV1 $^{-/-}$ (Caterina et al., 2000).

Animals were intercrossed to generate experimental animals (of either sex) as described in the text; the TRPV1-DTR and TRPM8-DTR were hemizygous and Mrgprd-DTR heterozygous in all experiments. For diphtheria toxin mediated cell ablation we began administering DT in adult mice (> 5 weeks old); behavioral testing began at least 1 week and no more than 2 months after DT administration using mice weighing 20–30g (2–4 month old). Over this 2 month time period, repeat experiments established that phenotypes were completely stable. Control mice for each experimental series were DT treated non-transgenic littermates and were co-housed with DTR-siblings. We tested mice in behavioral assays before recording its ear-tag number, thus experimenters were blind to the genotype of individual mice. No significant differences in behavior between different sets of controls were noted and for simplicity data was combined from the TRPV1-DTR and TRPM8-DTR littermate controls unless otherwise specified.

We experimentally established the effectiveness of DT mediated ablation in the different lines and assessed the minimal dosage required to achieve > 95 % elimination of target cells. For TRPM8-DTR animals, this required daily injection of DT (100ng) for 7 days; for TRPV1-DTR and Mrgprd-DTR animals, cell elimination was achieved by regular injection of 200ng DT (administered for 5 days, followed by 2 days off, for 3 weeks); double and triple mutants all received this higher DT dose. We used the TRPV1-DTR line to check that DT indeed ablates cells from the ganglia by counting neurons in serial sections through the entire L4 ganglion of control (8660 ±667 NeuN positive neurons) and DT treated TRPV1-DTR mice (5760 ±467 NeuN positive neurons; significant difference between groups; $P< 0.01$, Student's t test, means ±s.e.m. (n=4). As expected approximately 33 % of total neurons are lost, consistent with selective loss of TRPV1-expressing neurons (Caterina et al., 2000). Core body temperature (Table 1) was measured using a rectal probe (Fine Science Tools); measurement was performed on 3 separate days.

Behavioral Assays

Chemical sensitivity—Eye wipe assays were performed to investigate the afferent functions of the ophthalmic branch of the trigeminal nerve. Capsaicin induced eye-wipes were counted for 1 minute following delivery of 50 μl of solution (50 μM capsaicin), PBS elicited eye wipes were subtracted from those measured for capsaicin. Wet-dog shakes were induced by i.p. injection of 50 mg/Kg icilin (Sigma) and numbers of whole body shakes were counted over 30 minutes as described (Dhaka et al., 2007).

Thermal preference—A two choice temperature assay was employed to determine thermal preference; mice were placed in an apparatus that had a fixed reference plate set at 25, 30, or 45 °C and a test-plate whose temperature was adjusted between 0 °C and 50 °C (T2CT, Bioseb, France). Each animal was tested twice for each set of parameters; the first assay was initiated by placement of the mouse on the plate set at the test-temperature, in the second assay the initial placement was reversed so that the mouse was placed on the fixed-plate. Mouse position was tracked over 5 minutes using an automated tracking system (Bioseb, France). Only assays in which mice sampled both plates within the first minute were scored.

Thermal reflex response assays were used to assess acute temperature sensitivity. For hot responses, mice were placed on a plate at 55 °C and latency to display withdrawal of hind limbs was measured; cut-off to prevent injury was set at 30 seconds. A cold plantar assay (Brenner et al., 2012) was used to assess sensitivity to low temperatures; for this assay, animals were habituated (> 15 min) in individual chambers with a 1/16 inch glass plate floor and a dry ice pellet was applied below the hind-paw. The time for paw-withdrawal was measured; for each reflex-response assay, animals were tested more than 3 times.

In situ hybridization and Immunohistochemistry

In situ hybridization (ISH) was performed at high stringency (washed 30 min, 0.2x SSC, 70 °C) as described previously (Hoon et al., 1999). ISH of molecular markers were performed on tissue from >5 transgenic and control mice. Serial sections from >10 sections per mouse were hybridized and numbers of cells counted in order to quantize numbers of neurons. Immunohistochemistry was performed with chicken anti-GFP (1:1000 Abcam), and rabbit anti NeuN (1:800 Abcam) and developed with donkey anti-chicken Alexa488 or donkey anti-rabbit Alexa 488 (Jackson Immunolabs) respectively. Images were collected using a Microphot FX microscope (Nikon) and images were processed with Adobe Photoshop.

Results

Selective ablation of TRPV1 and TRPM8-neurons

Previously, our attempt to ablate the thermoreceptor neurons expressing TRPV1 with constitutively expressed DTA was frustrated because TRPV1 is expressed in the precursors of several functionally distinct classes of nociceptor (Mishra et al., 2011). Here we adopted a different strategy that combined molecular genetic targeting and toxin injection to selectively kill defined classes of these somatosensory neurons in adult mice. This approach makes use of the fact that mice are normally insensitive to diphtheria toxin (DT) because they do not express a receptor that mediates cellular uptake of the active toxin. This means that cell-specific DT-sensitivity can be achieved by directed expression of the human diphtheria toxin receptor (DTR) (Saito et al., 2001). Thus to target putative thermoreceptive neurons, we engineered BAC-transgenic mice that express DTR the under the control of TRPV1- and TRPM8-regulatory sequences (see Figure 1a) and used DT injection in adult mice to selective ablate these classes of somatosensory neurons. In situ hybridization studies (Figure 1b and Figure 2a) indicated that TRPV1-neurons are effectively and selectively killed by DT-injection in mice expressing the TRPV1-DTR transgene and that TRPM8-neurons (but not other classes of nociceptor) are eliminated in TRPM8-DTR mice. For simplicity, we refer to the toxin treated animals as TRPV1-DTR and TRPM8-DTR mice respectively.

Given that approximately one third of somatosensory neurons express TRPV1, we expected that ablation of this class of somatosensory neuron should result in a dramatic reduction in the total neural count. Indeed, we found toxin treatment reduced the number of NeuN positive neurons by about 30 % in the DRG of TRPV1-DTR mice (see Methods for details). Analysis of interneurons in the spinal cord (Figure 3) revealed no noticeable differences between mutant and control animals indicating that TRPV1-cell ablation destroys peripheral input without grossly affecting the somatosensory circuitry. We also noted that core body temperature of TRPV1-DTR mice remained indistinguishable from that of controls (Table 1). Finally, we assayed capsaicin induced eye-wipes as a high sensitivity behavioral assay for TRPV1 function and observed no response in TRPV1-DTR animals (Figure 2b), while responses to the TRPM8-agonist icilin remained indistinguishable from those of toxin treated wild-type controls (Figure 2c). Thus TRPV1-DTR animals have a highly selective and essentially complete loss of TRPV1-expressing neurons.

TRPM8 is expressed in a much smaller subset of neurons than TRPV1 (see Figure 1b). Thus no significant differences in the number of DRG-neurons were detected in TRPM8-DTR mice. However, as expected, behavioral assays showed that these animals are completely unresponsive to the potent TRPM8-agonist, icilin (Figure 2c) while they retain responses to capsaicin that were indistinguishable from controls (Figure 2b). Like the TRPV1-DTR mice, TRPM8-DTRs exhibited completely normal expression of markers of spinal cord interneurons (Figure 3) and core body temperature (Table 1).

We next used a two-plate choice assay to assess temperature preference in TRPV1- and TRPM8-DTR animals. Controls included toxin treated littermates not carrying the transgenes as well as TRPV1$^{-/-}$ and TRPM8$^{-/-}$ mice. In essence, the assay allows us to set the temperature of one plate at a permissive temperature (fixed-plate) and to study the response of individual mice to changes in temperature of the other plate (test-plate) over a wide and physiologically relevant range (from 0 – 50 °C). When the fixed-plate was set at 30 °C, control mice showed a clear preference for the test-plate at 35 °C but exhibited increasingly strong avoidance of the test-plate when the temperature was raised or lowered outside the 25 – 40 °C range (Figure 4, open circles). Notably, TRPV1$^{-/-}$ mice behaved exactly like control animals in this assay (Figure 4a, pink circles). In contrast, DT-mediated ablation of TRPV1-neurons completely eliminated aversion to elevated temperatures (40 – 50 °C), but did not affect avoidance of cold (Figure 4a, red squares) highlighting the role of TRPV1-cells (but not TRPV1-itself) as selectively tuned hot sensors. The opposite phenotype was observed with TRPM8-DTR animals, which exhibited normal aversion to hot temperatures but a striking loss of response to cool and cold (Figure 4b, blue squares). Our data [and previous studies (Bautista et al., 2007, Colburn et al., 2007, Dhaka et al., 2007)] showed a qualitatively similar phenotype for TRPM8 $^{-/-}$ mice, with less aversion to cool (15 – 25 °C) temperatures than control mice (Figure 4b, pale blue circles, $P < 0.05$, Students t-test). However, ablation of TRPM8-cells resulted in a significantly stronger cold (0 – 15 °C) deficit than knockout of the TRPM8 gene (Figure 4b). Taken together our results confirm the hypothesis that TRPV1 and TRPM8-neurons provide selective, aversive input in response to heat and cold respectively and demonstrate that each of these classes of neurons utilize unknown thermosensors in addition to either TRPV1 or TRPM8.

A role for TRPA1 in cold detection?

One prominent, but controversial, candidate cold-sensor is the TRP-channel, TRPA1 (Story et al., 2003, Jordt et al., 2004, Bautista et al., 2006, Kwan et al., 2006, Caspani and Heppenstall, 2009, Karashima et al., 2009, Knowlton et al., 2010). Here, we demonstrate that TRPA1 is not expressed in the cold sensing TRPM8 cells but rather is exclusively found in TRPV1 expressing cells (Figure 2) confirming previous reports (Story et al., 2003, Mishra and Hoon, 2010). Interestingly, we noted that TRPM8-DTR mice exhibited modest but appreciable aversion to 0 °C, the lowest temperature tested in the 2-place assay (Figure 4b). Thus we hypothesized that TRPA1-input (and TRPV1-cells) might be involved in triggering aversion to painful cold in TRPM8-DTR mice.

To test this hypothesis, we first confirmed that TRPA1-expression is eliminated in TRPV1-DTR mice (Figure 5a and 2a). Next we used a cold plantar assay (Brenner et al., 2012) to investigate whether TRPV1-cell input affects an animal's tolerance of noxious cold. In this assay, paw withdrawal latency is measured following application of a piece of dry ice to the glass plate directly under the mouse's hind paw (rapid cooling of the plantar surface). As expected knockout of TRPM8 or ablation of TRPM8 cells significantly increased the time it took for mice to respond (Figure 5b), and in agreement with the two-plate preference assay, ablation of the TRPM8 cells resulted in a more profound deficit in cold sensing than was observed in TRPM8$^{-/-}$ mice. In contrast, TRPV1-DTR mice displayed reaction times that were no different from control animals and more importantly, TRPV1-DTR/TRPM8-DTR animals were indistinguishable in their response from TRPM8-DTR animals (Figure 5b). Therefore TRPA1 has no major role in this type of cold reflex responses.

Painful temperatures activate Mrgprd-expressing neurons

Which cells mediate the residual responses to cold in TRPM8-DTR animals? Previously we have shown that TRPV1-DTA mice, which lack several classes of sensory neuron because the TRPV1-lineage is ablated during development in this strain, were completely insensitive

J Neurosci. Author manuscript; available in PMC 2013 September 27.

to cold in the two plate assay (Mishra et al., 2011). Therefore we suspected that other nociceptors that are lost in TRPV1-DTA mice might be involved and set out to examine if Mrgprd-expressing neurons augment cold sensation through TRPM8 neurons at very low temperatures. We obtained the previously reported Mrgprd-DTR knockin-line (Cavanaugh et al., 2009) (a generous gift from David Anderson) and confirmed that these mice have no significant cold sensing phenotype (see Figure 6b, light gray bar). Next we crossed the Mrgprd-DTR allele into a TRPM8-DTR background to ablate both sets of neurons (Figure 6a) and found that these mice exhibited significantly less cold sensitivity than TRPM8-DTR mice in the cold plantar assay of noxious cold (Figure 6b, dark gray bar). Thus Mrgprd-cells not only detect mechanical pain (Cavanaugh et al., 2009) but also respond to extreme cold. Nonetheless, the TRPV1-DTA mice that we described previously (Mishra et al., 2011) have an even greater deficit in cold sensation than the Mrgprd-DTR/TRPM8-DTR double mutants implying that additional TRPV1-lineage cells may also respond to painfully cold temperatures. Could functional responses of TRPV1-cells to cold, unmasked by ablating both TRPM8 and Mrgprd-neurons, account for this difference? To answer this question we generated and tested triple mutants lacking TRPV1-, TRPM8- and Mrgprd-neurons. These mice (Figure 6b, hatched bar) were indistinguishable in their response to cold to the double mutants that still had normal TRPV1- input (Figure 6b, dark grey bar). Thus our data demonstrate that TRPV1-cells play a very minor role (if any) in cold detection and since TRPA1 is exclusively expressed in TRPV1-cells, further limit the role this protein can have as a cold detector.

We also examined responses of mutant animals to painful heat. Because, exposure to temperatures above 50 °C needs to be strictly limited to prevent injury, we could not use the two-place preference test and instead used paw withdrawal from a 55 °C heated block with a 30 s exposure maximum to assay noxious heat. As shown previously (Mishra et al., 2011) TRPV1-DTA mice exhibit no escape reaction within the cutoff (Figure 7b, black bar). However, although TRPV1-DTR mice are far less sensitive to heat than controls, they still exhibit paw-withdrawal within the 30 s cutoff (Figure 7b, red bar). Do Mrgprd cells contribute to responses to painful heat as well as noxious cold? Again we generated double mutant animals, this time TRPV1-DTR/Mrgprd-DTR, demonstrated efficient ablation of both populations of sensory neurons (Figure 7a) and tested behavioral responses. Importantly, just as observed for cold sensation, ablation of Mrgprd cells alone had no effect on paw withdrawal latency, but when combined with ablation of TRPV1-cells, it significantly increased reaction time to high temperature (Figure 7b). Indeed TRPV1-DTR/Mrgprd-DTR mice were almost completely insensitive to 55 °C exposure just like the TRPV1-DTA animals. Taken together these results show that Mrgprd neurons respond to extremes of heat and cold and may enhance escape reactions (as well as modify the sensory experience) to these noxious and painful stimuli.

Attraction to warmth is mediated by TRPV1 and TRPM8-cells

Interestingly, TRPM8-DTR mice not only showed reduced responses to cold temperatures in the two-plate preference assay, but also a marked shift in their preferred temperature from 35 °C in control animals to 25 °C in the mutants (Figure 4). Thus we set out to investigate how TRPV1- and TRPM8-neurons affect the perception of warmth (i.e. the temperature range that a normal mouse actively seeks out). To do this, we again used the two plate assay but initially decreased the temperature of the fixed-plate to 25 °C, the preference maximum for TRPM8-DTR mice (Figure 4). Under these conditions control mice preferred to remain on the warmer block at temperatures up to and including 40 °C but still strongly disliked higher temperatures (Figure 8a). TRPV1-DTR animals behaved indistinguishably from controls in the warm (30 – 40 °C) regime but unlike controls, continued to show increased preference for the test-plate even temperatures that are normally strongly aversive (up to 50

°C, Figure 8a, red squares). As predicted TRPM8-DTR mice exhibited a completely different phenotype. These animals never were attracted to the warmer environment and importantly showed progressively increasing aversion at 35 °C and above (Figure 8a).

We also subjected animals to the opposite scenario where the fixed-block was set at a modestly aversive elevated temperature (45 °C). In this assay, normal mice chose to stay at cooler test temperatures far longer than when the fixed-block is set at 30 °C (compare Figure 8b and Figure 4). As expected, TRPV1-DTRs showed strong preference for 45 °C at all temperatures tested (Figure 8b) while TRPM8-DTR mice always preferred the colder temperature even when the test-block was at 0 °C (Figure 8b, blue squares). Taken together these data indicate that the threshold for TRPM8-mediated aversion is around 35 °C (body temperature), and suggest that TRPV1 and TRPM8 neurons provide all thermosensitive input from the periphery. If this is true, then the simplest explanation for the sensation of warmth would be that it reflects the absence of aversive signaling from either cold or hot sensing neurons rather than a positive signal from another class of thermoreceptor. This model predicts that ablation of both hot and cold sensing neurons should yield mice that cannot detect warmth. Figure 8 (panels c & d) demonstrates that TRPV1-DTR/TRPM8-DTR animals fail to exhibit attraction to temperatures that normal animals actively seek out and indeed are essentially indifferent to temperatures between 0 and 50 °C. Thus input through the TRPM8 and TRPV1 cells not only alert mice to unpleasant or noxious temperatures but also specify their preferred temperature range and control responses to warmth.

Discussion

Here, we used a selective ablation strategy to explore the contribution of individual, molecularly defined classes of sensory neuron to the mammalian sense of temperature. Importantly, the DTR-transgenes that we developed are very selectively expressed in sensory neurons (see Methods for details) and ablation of each class of cell was specific and had no detectable effects on other sensory neurons (Figure 1 & 2) or the wider somatosensory circuitry (Figure 3). Moreover, diphtheria-toxin treatment of the different transgenic mice resulted in reproducible, highly selective and non-overlapping effects on animal behavior ruling out non-specific effects of this experimental technique. Indeed, similar approaches have been extensively validated in previous studies of the peripheral nervous system (Gogos et al., 2000, Cavanaugh et al., 2009), CNS (Luquet et al., 2005), and for other sensory systems (Huang et al., 2006). Thus our results demonstrate that thermosensation almost exclusively uses two differentially tuned populations of sensory neurons: TRPV1 cells that respond to high temperature and TRPM8 cells that detect cold to encode a full range of sensory percepts (cold, cool, warm and hot). Interestingly, thermoreception in fruit flies closely parallels its mammalian counterpart and also uses separate populations of hot and cold responsive neurons to encode the full temperature range (Gallio et al., 2011), perhaps reflecting a common evolutionary need of animals to avoid temperature extremes.

In our experiments, ablating TRPV1-cells but not knockout of the TRPV1-channel itself had major effects on an animal's ability to detect elevated temperatures as high as 50 °C implying that these cells must express novel heat sensors in addition to TRPV1. Similarly, although TRPM8 serves as an important receptor for cooling (Bautista et al., 2007, Colburn et al., 2007, Dhaka et al., 2007) the greater thermal deficit of TRPM8-DTR mice compared to knockouts (Figures 4 & 5) indicates that additional cold receptors are activated in these neurons when temperatures fall below 15 °C. Our data also demonstrate that extremes of heat and cold recruit at least one more class of nociceptors, the cells expressing Mrgprd, which are not normally thought to respond to thermal cues (Cavanaugh et al., 2009). While it is possible that these neurons in fact are a class of thermosensor that only respond to

temperature extremes we favor a model where the known sensitivity of Mrgprd-nociceptors to inflammatory and algesic compounds (Dussor et al., 2008, Rau et al., 2009) accounts for their recruitment by both noxious heat and noxious cold. In contrast, no role for TRPV1-cells (and by implication the TRPA1 channel) was detected in responses to low temperatures (Figures 4 – 6).

Are TRPV1 and TRPA1 required for any types of thermal response? Our study was primarily designed to uncover the role of specific cells types in somatosensation rather than the role of specific receptors. Nonetheless, we found little evidence that either channel is directly involved in detecting thermal cues over a wide, physiologically relevant temperature range. Notably our data are largely consistent with previous reports (Caterina et al., 2000, Bautista et al., 2006) in the temperature range (0 – 50 °C) that we studied. Indeed, the biggest effects of TRPV1 on heat detection appear at more extreme temperatures and after injury or inflammation (Caterina et al., 2000), conditions not investigated in our study. Similarly, TRPA1 may have a more significant role as a cold transducer after injury (del Camino et al., 2010) and it is possible that cellular coding is also modified in these circumstances. However, in normal mice TRPA1 neither affects temperature preference nor withdrawal responses from cold thus challenging the notion (Kwan et al., 2006, Karashima et al., 2009) that TRPA1 is a cold-temperature sensor in the absence of injury.

Interestingly ablation of TRPM8-neurons caused major temperature detection deficits in the warm temperature range (up to 35 °C, Figures 4 & 8) in line with previous in vivo studies (de la Pena et al., 2005, Madrid et al., 2009, Sarria et al., 2012) that found similar tuning using functional modulation of these cells. Together with the flat temperature response profile of TRPV1-DTR/TRPM8-DTR double mutant animals, the most parsimonious explanation for the feeling of warmth is that this percept actually reflects minimal signaling through the TRPM8-cells (and little or no input from TRPV1-cells). However, because TRPV1-expressing neurons (and TRPM8-counterparts) may not be homogeneous populations of thermosensors, we cannot completely exclude more complex scenarios e.g., where a subset of TRPV1-neurons is tuned to respond to warm temperatures and provides attractive rather than aversive input.

How would the simple model (lack of aversive input through TRPM8 and TRPV1 neurons) fit with our own experience? While it is practically impossible to extrapolate human perception to behavioral studies of mice, it remains likely that a very similar peripheral mechanism accounts for temperature detection in both species. The separate inputs of cooling and heating nicely explain our ability to distinguish cold from hot and why inflammation and injury, which increase responses through TRPV1-cells (Caterina and Julius, 2001), make normally pleasant heat unbearable. Other familiar experiences like the soothing effects of a cool breeze or menthol as well as the thermal grill illusion (Craig, 2002) or the painful effects of going from a freezing environment to a warm one point to significant and complex interactions between these two distinct sensory lines. Finally, our evaluation of temperature (and the valence assigned to stimuli) can vary greatly according to our core-temperature. Thus we anticipate that future studies mapping the connections and circuitry of the afferent TRPM8 and TRPV1 sensory lines will be useful in explaining how sensation drives temperature perception and how this information interacts with internal and emotional state to control behavior.

References

Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell. 2006; 124:1269–1282. [PubMed: 16564016]

Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D. The menthol receptor TRPM8 is the principal detector of environmental cold. Nature. 2007; 448:204–208. [PubMed: 17538622]

Brenner DS, Golden JP, Gereau RWt. A novel behavioral assay for measuring cold sensation in mice. PLoS One. 2012; 7:e39765. [PubMed: 22745825]

Caspani O, Heppenstall PA. TRPA1 and cold transduction: an unresolved issue? The Journal of general physiology. 2009; 133:245–249. [PubMed: 19237589]

Caterina MJ, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci. 2001; 24:487–517. [PubMed: 11283319]

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science. 2000; 288:306–313. [PubMed: 10764638]

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997; 389:816–824. [PubMed: 9349813]

Cavanaugh DJ, Chesler AT, Jackson AC, Sigal YM, Yamanaka H, Grant R, O'Donnell D, Nicoll RA, Shah NM, Julius D, Basbaum AI. Trpv1 reporter mice reveal highly restricted brain distribution and functional expression in arteriolar smooth muscle cells. J Neurosci. 2011; 31:5067–5077. [PubMed: 21451044]

Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A. 2009; 106:9075–9080. [PubMed: 19451647]

Colburn RW, Lubin ML, Stone DJ Jr, Wang Y, Lawrence D, D'Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N. Attenuated cold sensitivity in TRPM8 null mice. Neuron. 2007; 54:379–386. [PubMed: 17481392]

Craig AD. How do you feel? Interoception: the sense of the physiological condition of the body. Nat Rev Neurosci. 2002; 3:655–666. [PubMed: 12154366]

de la Pena E, Malkia A, Cabedo H, Belmonte C, Viana F. The contribution of TRPM8 channels to cold sensing in mammalian neurones. J Physiol. 2005; 567:415–426. [PubMed: 15961432]

del Camino D, Murphy S, Heiry M, Barrett LB, Earley TJ, Cook CA, Petrus MJ, Zhao M, D'Amours M, Deering N, Brenner GJ, Costigan M, Hayward NJ, Chong JA, Fanger CM, Woolf CJ, Patapoutian A, Moran MM. TRPA1 contributes to cold hypersensitivity. J Neurosci. 2010; 30:15165–15174. [PubMed: 21068322]

Dhaka A, Murray AN, Mathur J, Earley TJ, Petrus MJ, Patapoutian A. TRPM8 is required for cold sensation in mice. Neuron. 2007; 54:371–378. [PubMed: 17481391]

Dussor G, Zylka MJ, Anderson DJ, McCleskey EW. Cutaneous sensory neurons expressing the Mrgprd receptor sense extracellular ATP and are putative nociceptors. J Neurophysiol. 2008; 99:1581–1589. [PubMed: 18234974]

Gallio M, Ofstad TA, Macpherson LJ, Wang JW, Zuker CS. The coding of temperature in the Drosophila brain. Cell. 2011; 144:614–624. [PubMed: 21335241]

Gogos JA, Osborne J, Nemes A, Mendelsohn M, Axel R. Genetic ablation and restoration of the olfactory topographic map. Cell. 2000; 103:609–620. [PubMed: 11106731]

Hoon MA, Adler E, Lindemeier J, Battey JF, Ryba NJ, Zuker CS. Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. Cell. 1999; 96:541–551. [PubMed: 10052456]

Huang AL, Chen X, Hoon MA, Chandrashekar J, Guo W, Trankner D, Ryba NJ, Zuker CS. The cells and logic for mammalian sour taste detection. Nature. 2006; 442:934–938. [PubMed: 16929298]

Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Hogestatt ED, Meng ID, Julius D. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature. 2004; 427:260–265. [PubMed: 14712238]

Jordt SE, McKemy DD, Julius D. Lessons from peppers and peppermint: the molecular logic of thermosensation. Curr Opin Neurobiol. 2003; 13:487–492. [PubMed: 12965298]

Karai L, Brown DC, Mannes AJ, Connelly ST, Brown J, Gandal M, Wellisch OM, Neubert JK, Olah Z, Iadarola MJ. Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. J Clin Invest. 2004; 113:1344–1352. [PubMed: 15124026]

Karashima Y, Talavera K, Everaerts W, Janssens A, Kwan KY, Vennekens R, Nilius B, Voets T. TRPA1 acts as a cold sensor in vitro and in vivo. Proc Natl Acad Sci U S A. 2009; 106:1273–1278. [PubMed: 19144922]

Knowlton WM, Bifolck-Fisher A, Bautista DM, McKemy DD. TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics in vivo. Pain. 2010; 150:340–350. [PubMed: 20542379]

Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron. 2006; 50:277–289. [PubMed: 16630838]

Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics. 2001; 73:56–65. [PubMed: 11352566]

Luquet S, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. Science. 2005; 310:683–685. [PubMed: 16254186]

Madrid R, de la Pena E, Donovan-Rodriguez T, Belmonte C, Viana F. Variable threshold of trigeminal cold-thermosensitive neurons is determined by a balance between TRPM8 and Kv1 potassium channels. J Neurosci. 2009; 29:3120–3131. [PubMed: 19279249]

McKemy DD, Neuhausser WM, Julius D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature. 2002; 416:52–58. [PubMed: 11882888]

Mishra SK, Hoon MA. Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. Mol Cell Neurosci. 2010; 43:157–163. [PubMed: 19853036]

Mishra SK, Tisel SM, Orestes P, Bhangoo SK, Hoon MA. TRPV1-lineage neurons are required for thermal sensation. EMBO J. 2011; 30:582–593. [PubMed: 21139565]

Park U, Vastani N, Guan Y, Raja SN, Koltzenburg M, Caterina MJ. TRP vanilloid 2 knock-out mice are susceptible to perinatal lethality but display normal thermal and mechanical nociception. J Neurosci. 2011; 31:11425–11436. [PubMed: 21832173]

Patapoutian A, Peier AM, Story GM, Viswanath V. ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci. 2003; 4:529–539. [PubMed: 12838328]

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A. A TRP channel that senses cold stimuli and menthol. Cell. 2002; 108:705–715. [PubMed: 11893340]

Rau KK, McIlwrath SL, Wang H, Lawson JJ, Jankowski MP, Zylka MJ, Anderson DJ, Koerber HR. Mrgprd enhances excitability in specific populations of cutaneous murine polymodal nociceptors. J Neurosci. 2009; 29:8612–8619. [PubMed: 19571152]

Saito M, Iwawaki T, Taya C, Yonekawa H, Noda M, Inui Y, Mekada E, Kimata Y, Tsuru A, Kohno K. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. Nature biotechnology. 2001; 19:746–750.

Sarria I, Ling J, Xu GY, Gu JG. Sensory discrimination between innocuous and noxious cold by TRPM8-expressing DRG neurons of rats. Mol Pain. 2012; 8:79. [PubMed: 23092296]

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell. 2003; 112:819–829. [PubMed: 12654248]

Vriens J, Owsianik G, Hofmann T, Philipp SE, Stab J, Chen X, Benoit M, Xue F, Janssens A, Kerselaers S, Oberwinkler J, Vennekens R, Gudermann T, Nilius B, Voets T. TRPM3 is a nociceptor channel involved in the detection of noxious heat. Neuron. 2011; 70:482–494. [PubMed: 21555074]

Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM. Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci. 2004; 24:6410–6415. [PubMed: 15254097]

Woolf CJ, Ma Q. Nociceptors--noxious stimulus detectors. Neuron. 2007; 55:353–364. [PubMed: 17678850]

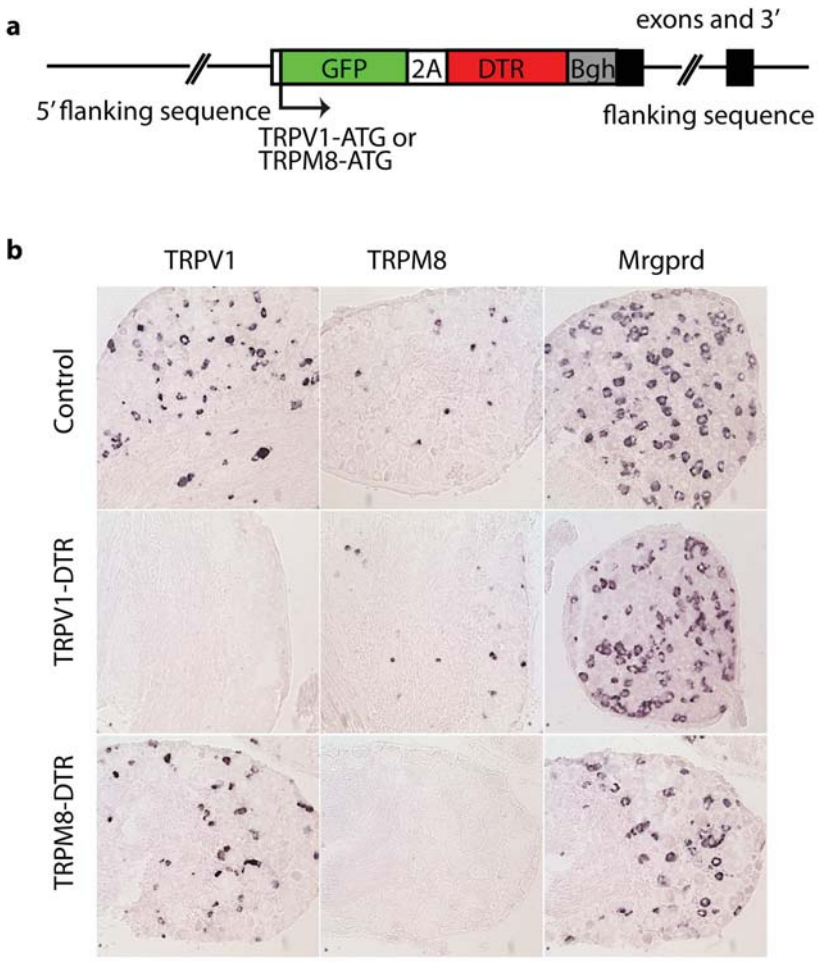


Figure 1. Ablation of TRPV1 and TRPM8-expressing cells

(a) To dissect the cells required for thermosensation we generated mice that carried BAC transgenes containing an insertion of GFP fused via a foot and mouth disease virus 2A-peptide to diphtheria toxin receptor (DTR) at the initiation codon of the TRPV1 or TRPM8 genes. These mice were treated with DT, to eliminate sensory neurons in adult animals. (b) ISH of sections through DRG demonstrates that ablation of TRPV1- and TRPM8-expressing neurons in DT treated TRPV1-DTR and TRPM8-DTR mice are selective for the targeted cells.

J Neurosci. Author manuscript; available in PMC 2013 September 27.

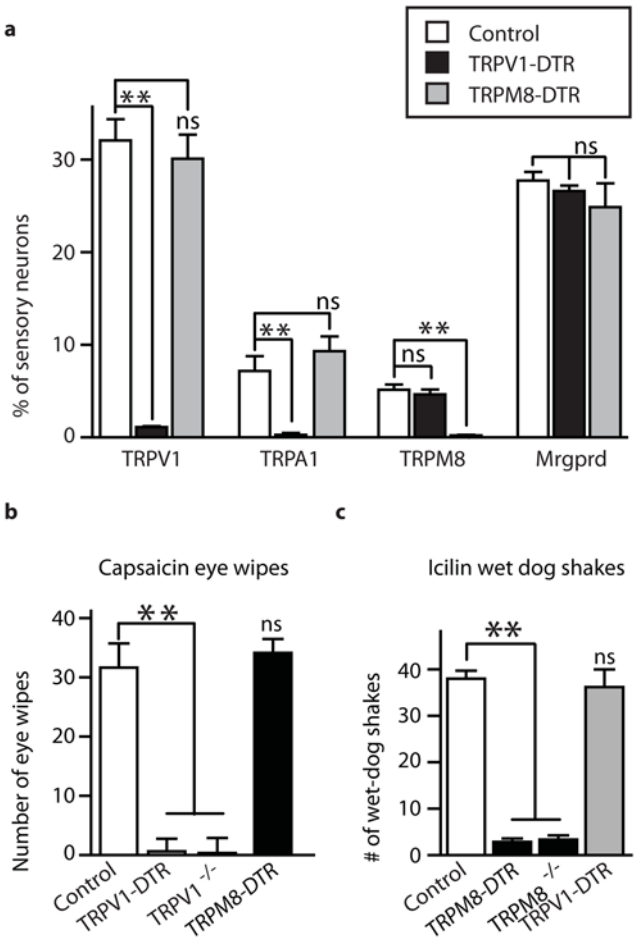


Figure 2. Specific ablation of sensory neurons is achieved by DT treatment of DTR-transgenic mice

(a) DT treatment of TRPV1- and TPRM8-DTR mice generates animals with specific loss of TRPV1- and TRPM8-expressing neurons. ISH of sections through DRG were probed with molecular markers as indicated. Lumbar DRGs from several different mutant mice were analyzed by ISH for expression of TRPV1, TRPA1, TRPM8 and Mrgprd; serial sections were stained for (NeuN+) to provide a total neuron count. Data represent the percentage of total neurons that were positive by ISH and are means ± s. e. m. (n=3); significance was assessed using Student's t test (** indicates $P< 0.01$). (b) Capsaicin elicited eye-wipes and (c) icilin induced wet dog shakes were used to assess loss of TRPV1 and TRPM8 function, respectively. (b) TRPV1-DTR and TRPV1 $^{-/-}$ but not TRPM8-DTR mice lose eye-wipe responses (** $P< 0.01$, Student's t test); in contrast (c) responses to icilin are lost in TRPM8-DTR and TRPM8 $^{-/-}$ but not TRPV1-mice (** $P< 0.01$, Student's t test). Data represent means ±s. e. m. ($n \geq 6$ animals).

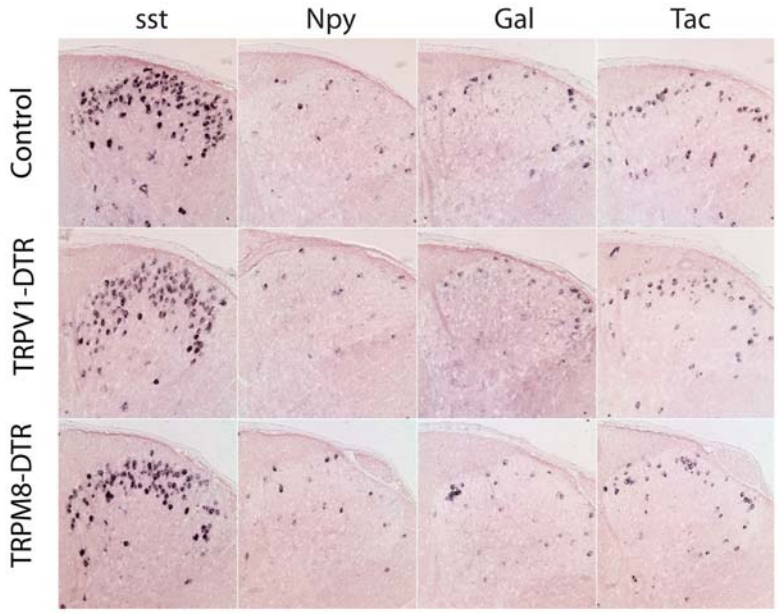


Figure 3. Ablation of TRPV1- and TRPM8-expressing sensory neurons does not alter numbers of spinal cord interneurons

ISH was performed with probes to different neuropeptides on sections through the dorsal horn of the spinal cord of control and mutant animals as indicated. No significant differences between genotypes were observed for numbers of positive neurons.

J Neurosci. Author manuscript; available in PMC 2013 September 27.

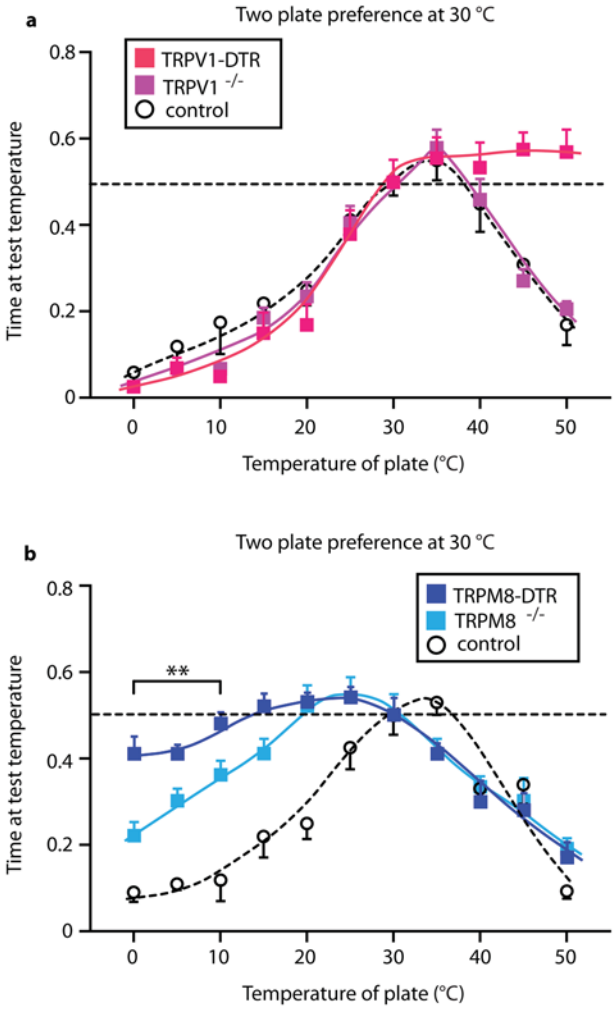


Figure 4. Elimination of TRPV1 and TRPM8-expressing neurons impair avoidance to heat and cold temperatures respectively

Two plate preference tests were used to assess thermal responses of TRPV1- and TRPM8-DTR mice. In these experiments the fixed-plate was set at 30 °C. (a) TRPV1-DTR mice (red squares) show no behavioral responses to hot temperatures (45 – 50 °C; $P < 0.01$, Student's t test) that are strongly aversive to control mice (TRPV1-DTR non-transgenic DT treated littermates; open circles). In contrast, TRPV1$^{-/-}$ animals (pink squares) exhibit responses that are indistinguishable from controls. (b) TRPM8-DTR mice (dark blue squares) have greatly attenuated responses to cold in comparison to controls (TRPM8-DTR non-transgenic DT treated littermates; open circles), but retain strong aversion to heat. Significant differences between genotypes were observed from 0 to 25 °C and at 35 °C ($P < 0.01$, Student's t test). TRPM8 $^{-/-}$ mutants (pale blue squares) are also less sensitive to cool temperatures (5–20 °C) than controls, but are significantly more responsive to cold (0–10 °C) than TRPM8-DTR mice (# $P < 0.05$, Student's t test). Time at test temperature represents the fraction of time mice spent on the test-plate versus the fixed-plate; data are means ± s. e. m. ($n \geq 6$ animals).

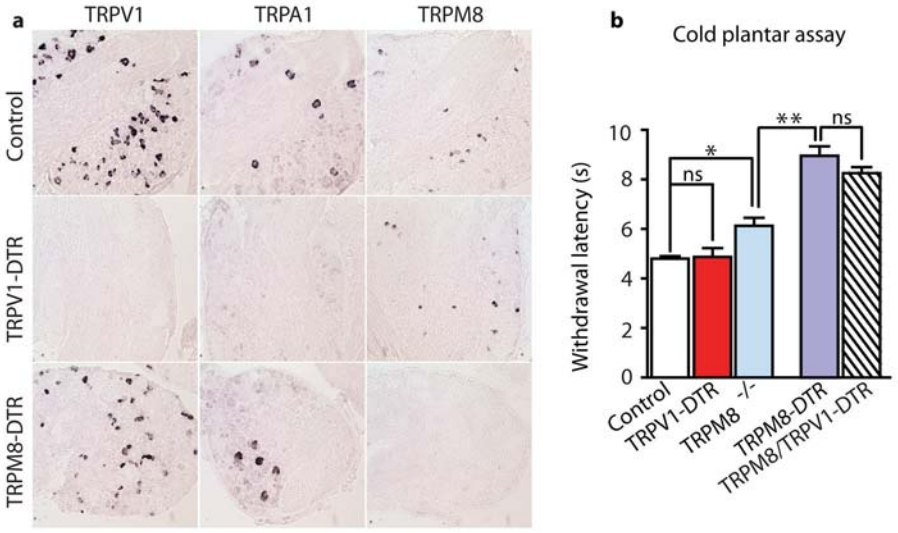


Figure 5. TRPA1-expressing neurons do not play a detectable role in cold reflex behavioral responses

(a) ISH of sections through DRG was used to examine TRPA1 expression; after ablation of TRPV1 neurons all TRPA1-expression was lost. (b) A cold-plantar assay was used to assess responses of various mutant mice to rapid cooling. In this assay, $TRPM8^{-/-}$ mutants (pale blue bar) are less sensitive than controls (* $P < 0.05$, Student's t test), but are significantly more responsive than TRPM8-DTR mice (** $P < 0.01$, Student's t test). In contrast, loss of TRPA1 input (elimination of TRPV1-cells) had no effect on paw withdrawal (TRPV1-DTR; red bar) even in mice where TRPM8-cold responsive cells were also killed (TRPV1-DTR/ TRPM8-DTR; hashed bar). Data represent means ± s. e. m. ($n \geq 6$ animals).

J Neurosci. Author manuscript; available in PMC 2013 September 27.

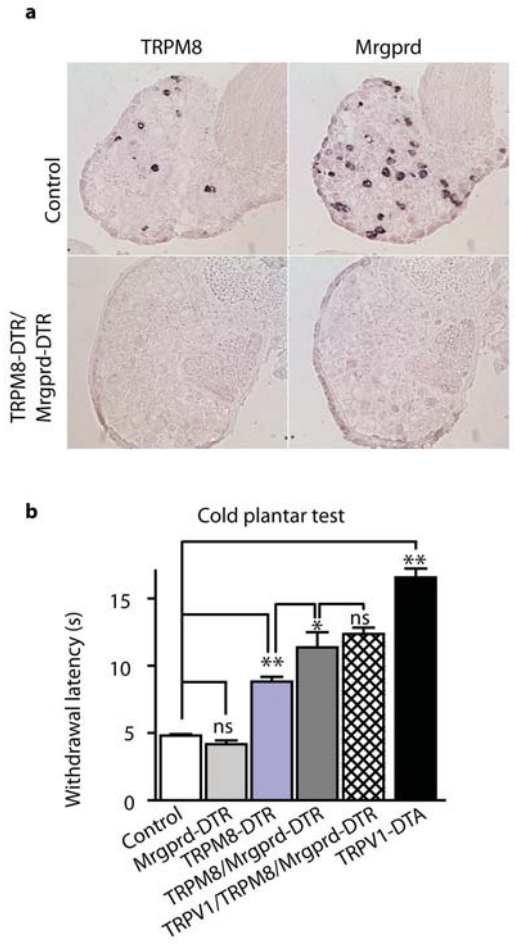


Figure 6. Mrgprd-neurons contribute to the detection of noxious cold

(a) Representative ISH of sections through DRG from TRPM8-DTR/Mrgprd-DTR mice illustrates selective loss of TRPM8 and Mrgprd expressing cells. (b) In cold plantar tests TRPM8-DTR mice (blue bars) have significantly longer withdrawal latencies than controls (** P< 0.01, Student's t test); in contrast behavioral responses of Mrgprd-DTR mice (grey bars) were indistinguishable from controls (ns, Student's t test). Combined loss of TRPM8- and Mrgprd-neurons (dark grey bars) causes a significantly greater cold deficit than ablation of TRPM8-cells alone (* P< 0.05, Student's t test). Elimination of TRPV1-cells in addition to the TRPM8 and Mrgprd cells (cross-hatched bar) had no additional effect on paw withdrawal latency. As expected, TRPV1-DTA mutants (mice with an extensive loss of TRPV1-lineage neurons, black bar) exhibit significantly reduced withdrawal latency compared to controls and other genotypes (** P< 0.01, Student's t test). Data represent means ±s. e. m. (n ≥ 6 animals).

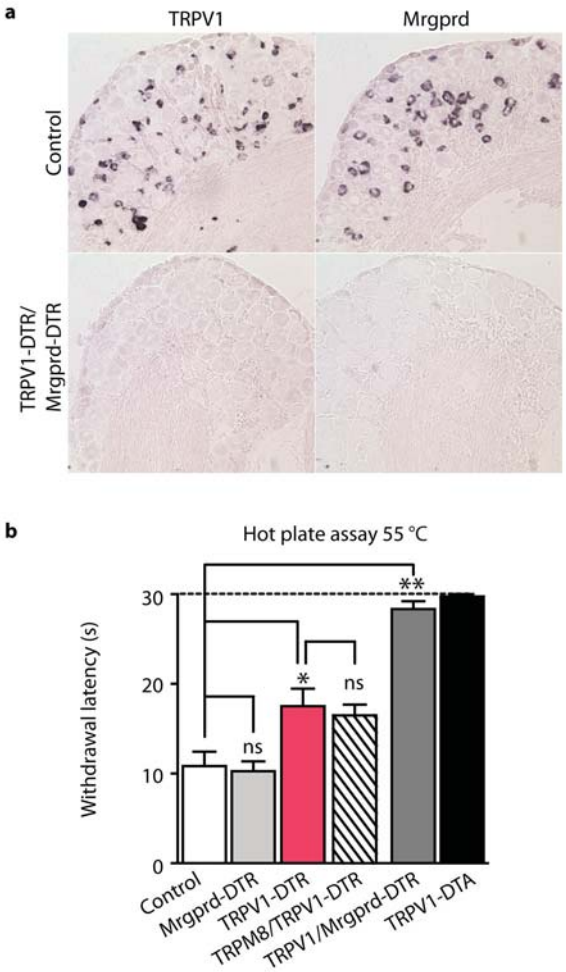


Figure 7. Mrgprd-neurons contribute to the detection of painful heat

(a) Representative ISH of sections through DRG from TRPV1-DTR/Mrgprd-DTR mice illustrates selective loss of TRPV1 and Mrgprd expressing cells. (b) A standard hot plate test that measures latency to show escape reactions to 55 °C was used to examine responses to noxious heat; significant differences between genotypes were assessed using Student's t test. Mrgprd-DTR mice (pale grey bar) display withdrawal responses indistinguishable from controls (open bar), whereas TRPV1-DTR mice (red bars), exhibit reduced response to noxious heat (* $P < 0.05$); additional ablation of TRPM8-cells (hatched bar) had no significant effect on response latency. In contrast, double mutants in which both the TRPV1- and Mrgprd-cells were eliminated (dark grey bar) and TRPV1-DTA mice that extensively lack both these classes of nociceptor (black bar) were essentially unresponsive within the cutoff for this assay. Data represent means ±s. e. m. ($n \geq 6$ animals).

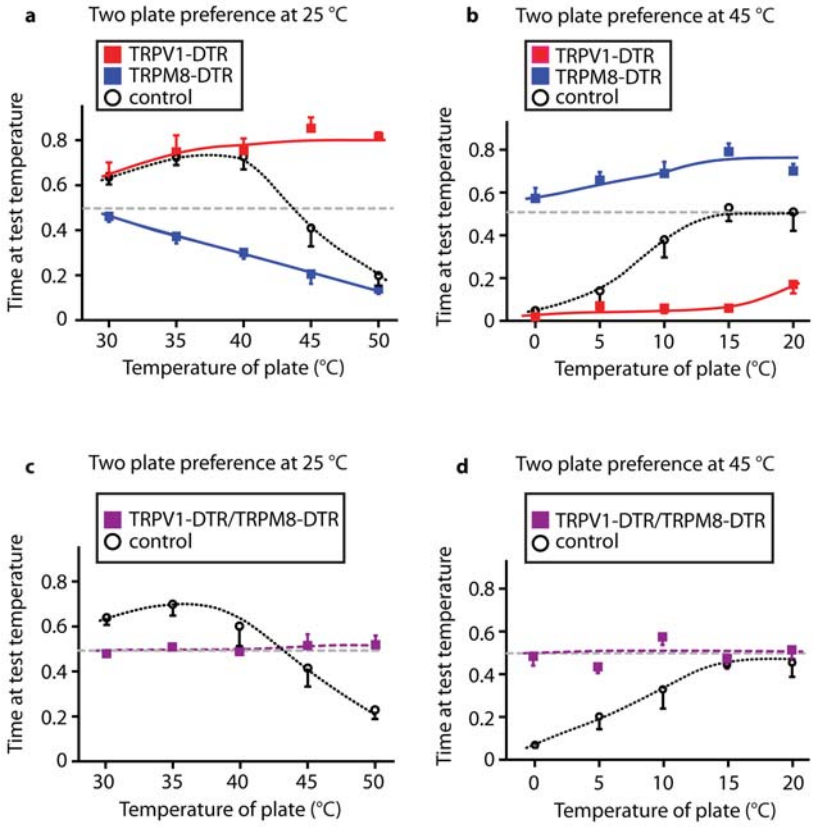


Figure 8. TRPV1- and TRPM8-neurons code for hot, cold and warm stimuli

(a) In two plate choice assays with the fixed-plate set at 25 °C, TRPV1-DTR mice (red squares) exhibit marked preference for test temperatures ≥ 40 °C, while control littermates (open circles) display profound avoidance to 45 - 50 °C. By contrast, TRPM8-DTR animals (blue squares) display no preference for warm and show increasing avoidance as temperature rises above 35 °C. (b) When the fixed-plate was set at 45 °C, TRPM8-DTR mice (blue squares) preferred all colder temperatures, while TRPV1-DTR animals (red squares) were significantly more averse to cold than controls (red squares) over the 10 – 20 °C temperature range ($P < 0.01$, Student's t test). (c, d) Mice with combined loss of TRPV1- and TRPM8-expressing cells (TRPV1-DTR/TRPM8-DTR, purple squares) were also tested using the two plate preference assay with the fixed-plate set at (c) 25 °C or (d) at 45 °C. The double mutant animals exhibited no temperature preference across the full range of temperatures. Time at test temperature represents the fraction of time mice spent on the test plate versus the control plate and data are means ±. m. ($n \geq 6$ animals).

Table 1

Body temperature of transgenic mice

Control	TRPV1-DTR	TRPM8-DTR	Mrgprd-DTR	TRPV1-DTR/TRPM8-DTR	TRPV1-DTR/TRPM8-DTR/Mrgprd-DTR
37.2 ± 0.32	37.3 ± 0.31	37.3 ± 0.40	37.3 ± 0.21	37.2 ± 0.33	37.1 ± 0.20

Body temperature (° C) was measured with a rectal probe on three consecutive days from 3 – 4 animals per genotype. Data represent means ± SD.

The EMBO Journal (2011) 30, 582–593 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

THE EMBO JOURNAL

TRPV1-lineage neurons are required for thermal sensation

Santosh K Mishra, Sarah M Tisel, Peihan Orestes, Sonia K Bhangoo and Mark A Hoon*

Molecular Genetics Unit, Laboratory of Sensory Biology, NIDCR, NIH, Bethesda, MD, USA

The ion-channel TRPV1 is believed to be a major sensor of noxious heat, but surprisingly animals lacking TRPV1 still display marked responses to elevated temperature. In this study, we explored the role of TRPV1-expressing neurons in somatosensation by generating mice wherein this lineage of cells was selectively labelled or ablated. Our data show that TRPV1 is an embryonic marker of many nociceptors including all TRPV1- and TRPM8-neurons as well as many Mrg-expressing neurons. Mutant mice lacking these cells are completely insensitive to hot or cold but in marked contrast retain normal touch and mechanical pain sensation. These animals also exhibit defective body temperature control and lose both itch and pain reactions to potent chemical mediators. Together with previous cell ablation studies, our results define and delimit the roles of TRPV1- and TRPM8-neurons in thermosensation, thermoregulation and nociception, thus significantly extending the concept of labelled lines in somatosensory coding.

The EMBO Journal (2011) **30,** 582–593. doi:10.1038/emboj.2010.325; Published online 7 December 2010
Subject Categories: signal transduction; neuroscience
Keywords: itch; pain; thermal; TRPM8; TRPV1

Introduction

Our senses provide an internal representation of aspects of the external world that are relevant to our survival, health and happiness. Among these, thermosensation provides animals with critical information about their environment and for us triggers perceptual responses that range from contentment to pain. Recent evidence suggests that a family of transient receptor potential (TRP)-related ion channels act in combination to detect environmental temperature (Jordt *et al*, 2003; Patapoutian *et al*, 2003). For example, the receptor for capsaicin (the 'hot' compound from chili peppers), TRPV1, is expressed in somatosensory neurons and thought to be the major mammalian sensor of noxious heat (Caterina *et al*, 1997). Similarly, TRPM8 is activated by menthol and cool temperatures (McKemy *et al*, 2002; Peier *et al*, 2002) while TRPV2 and TRPA1 have been suggested as sensors for extreme heat and cold, respectively (Caterina *et al*, 1999; Story *et al*, 2003; Jordt *et al*, 2004). In addition, TRPV3 (Moqrich *et al*, 2005) and TRPV4 (Lee and Caterina, 2005) have been reported to mediate warm responses although not by directly activating sensory neurons. Surprisingly, however, knockout mice lacking one or more of these TRP channels exhibit only modest deficits in temperature sensation (Caterina *et al*, 2000; Lee *et al*, 2005; Moqrich *et al*, 2005; Bautista *et al*, 2006, 2007; Kwan *et al*, 2006; Colburn *et al*, 2007; Dhaka *et al*, 2007) implying redundancy in the detection of heat and cold. In contrast, knockout of TRPV1 had greater effects on inflammatory pain sensation (Caterina *et al*, 2000) perhaps suggesting that its primary role may be as an integrator of several noxious signals rather than purely a thermosensor (Tominaga *et al*, 1998).

The problem of distinguishing and responding to different sensory modalities is often solved by selectively tuned receptors that are hardwired to trigger appropriate behaviour for example, the sweet- (attractive) versus bitter-(aversive)-labelled lines (Mueller *et al*, 2005). If a similar logic applies to somatosensation then removal of one class of neurons might eliminate specific sensory modalities without effecting responses to other types of stimuli. In contrast, if instead, stimuli are sensed by broadly tuned receptor neurons (e.g., multimodal nociceptors (Cain *et al*, 2001)), cellular ablation would never have such selective an effect.

Recently, resiniferatoxin-mediated killing (Mishra and Hoon, 2010) or capsaicin-induced deafferation (Cavanaugh *et al*, 2009) of TRPV1 neurons were shown to attenuate responses to heat but not to mechanically induced pain or cold. Moreover, ablation of a different subset of neurons expressing the mas-related G-protein-coupled receptor MrgD resulted in mechanosensory deficits (Cavanaugh *et al*, 2009) indicating that distinct cellular substrates function as sensors for different classes of noxious stimuli. These studies are important in defining the properties of specific subsets of sensory cells but silencing TRPV1 neurons was potentially incomplete and was relatively poorly defined (Cavanaugh *et al*, 2009; Mishra and Hoon, 2010). In addition, effects on mechanosensation were detected only when MrgD neurons were killed in adult animals but not if these cells were eliminated during development (Cavanaugh *et al*, 2009). Interestingly, diphtheria toxin-mediated killing of somatosensory neurons expressing the ion-channel Nav1.8 throughout development eliminated the majority of TRPV1 expression but had no effect on responses to heat (Abrahamsen *et al*, 2008) and instead altered mechanosensory responses and cold detection. Thus, there are indications that distinct classes of somatosensory neurons may selectively respond to particular sensory input but interpretation of results is limited by the ability to target the appropriate cells and redundancy or plasticity of the system. In this study, we used a molecular genetic approach to generate mice lacking all neurons in the TRPV1 lineage. Notably, these animals display no responses to thermal stimuli but retain normal proprioception and mechanosensation, thus delimiting

*Corresponding author. Molecular Genetics Unit, Laboratory of Sensory Biology, NIDCR, NIH, Building 49, Room 1A16, 49 Convent Drive, Bethesda, MD 20892, USA. Tel.: +1 301 402 6613; Fax: +1 301 480 3590; E-mail: mark.hoon@nih.gov

Received: 26 July 2010; accepted: 15 November 2010; published online: 7 December 2010

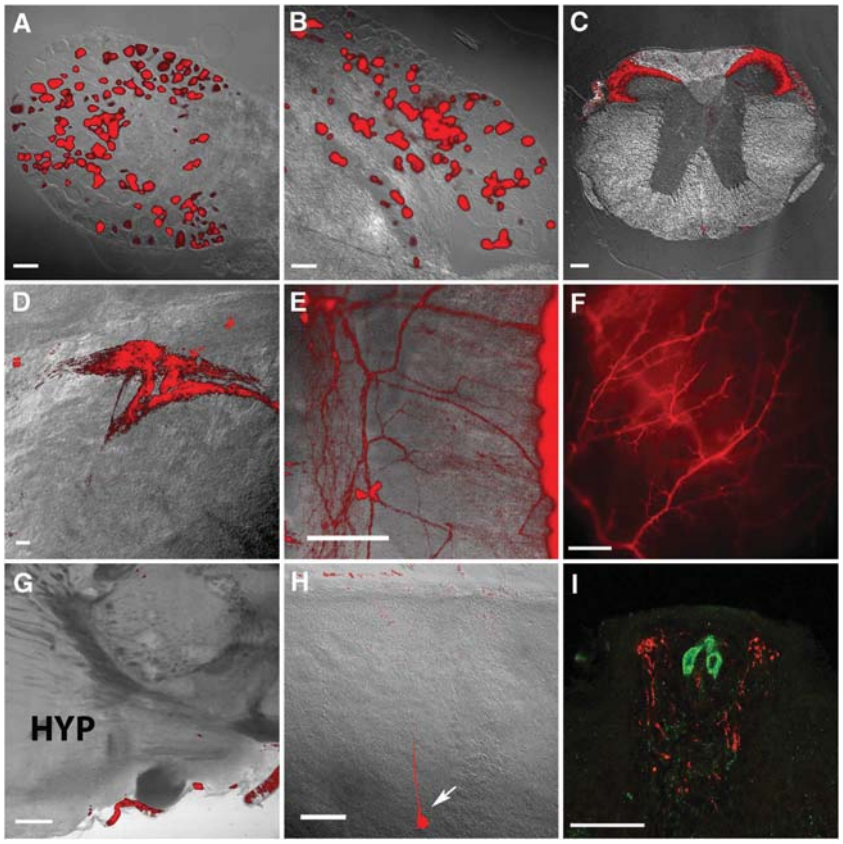


Figure 1 TRPV1-ai9 mice reveal the extent and limit of the TRPV1 lineage. Mice expressing tdTomato under the control of TRPV1-Cre-mediated recombination were perfused and tissue was removed for fluorescent imaging. Sections through the DRG (**A**) and trigeminal ganglion (**B**) reveal tdTomato expression in a subset of neurons that project to superficial lamina in the dorsal horn (**C**) and trigeminal tract (**D**), respectively. Whole-mount imaging of the cornea (**E**) and skin (**F**) illustrate the peripheral projections of these fibres. No fluorescent cell bodies were observed in the hypothalamus (HYP) (**G**) although widely scattered neurons in the cortex (**H**) were labelled. At the front of the tongue (**I**) fluorescent processes surround fungiform taste buds, highlighted by TRPM5 staining (green); however, taste receptor cells are not tdTomato positive. Scale bars: **A**, **B**, **D**, **I**, **H** 50 μm; **C** and **E** 100 μm; **F** and **G** 1 mm.

plasticity in the development of the thermosensory-labelled line. The mutant mice also exhibit markedly reduced ability to regulate their body temperature in response to a variety of challenges, demonstrating the importance of peripheral thermosensation in this homoeostatic process.

Results

We engineered BAC transgenic mice in which Cre recombinase was expressed under the control of TRPV1 (TRPV1-Cre, see Materials and methods and Supplementary Figure S1 for details). To examine the selectivity of Cre expression, TRPV1-Cre animals were crossed with various reporter lines including ai9 ROSA-stop-tdTomato mice (Madisen *et al*, 2010) to generate TRPV1-ai9 mice. Figure 1 demonstrates that, as expected, the dorsal root ganglia (DRG) and the trigeminal ganglia of TRPV1-ai9 mice contain tdTomato marked neurons. In addition, we saw labelling of cells in blood vessels (data not shown) but importantly only very few scattered fluorescent cells were observed outside these tissues for example, in the cortex (Figure 1H). Notably, other areas of the brain including the hypothalamus and hippocampus were devoid of labelling, as were the fungiform taste buds (Figure 1G, I and data not shown). Direct *In situ* hybridization (ISH) or immunohistochemistry also revealed no evidence for the expression of TRPV1 in the brain or taste tissue. Because of the intense fluorescence of tdTomato in these TRPV1-ai9 mice, peripheral and central projections of the labelled neurons were beautifully revealed (see Figure 1 for detail). We further characterized the specificity of Cre expression in the ganglia using double label ISH and probes that selectively recognize the native and transgene transcripts (see Materials and methods). There was complete correspondence of Cre and TRPV1 expression in adult tissue (Figure 2A) and Cre-mediated excision and TRPV1 in embryonic tissue (Supplementary Figure S2a).

TRPV1-DTA mice selectively lose thermal sensation

We crossed the TRPV1-Cre mice with a ROSA-stop-DTA line (Ivanova *et al*, 2005) to generate animals (TRPV1-DTA) in which a genetically specified population of sensory neurons was ablated. We note that the resulting TRPV1-DTA mice appear healthy and show no obvious phenotypic abnormalities highlighting the restricted nature of TRPV1 expression. For example, TRPV1-DTA animals do not show any signs of self-mutilation unlike mice in which the sciatic nerve is lesioned (Wall *et al*, 1979), there is no obvious change in wound healing (after skin burns or fight wounds) nor any deficit in taste responses (data not shown). In addition, analysis of markers of interneurons in the dorsal horn

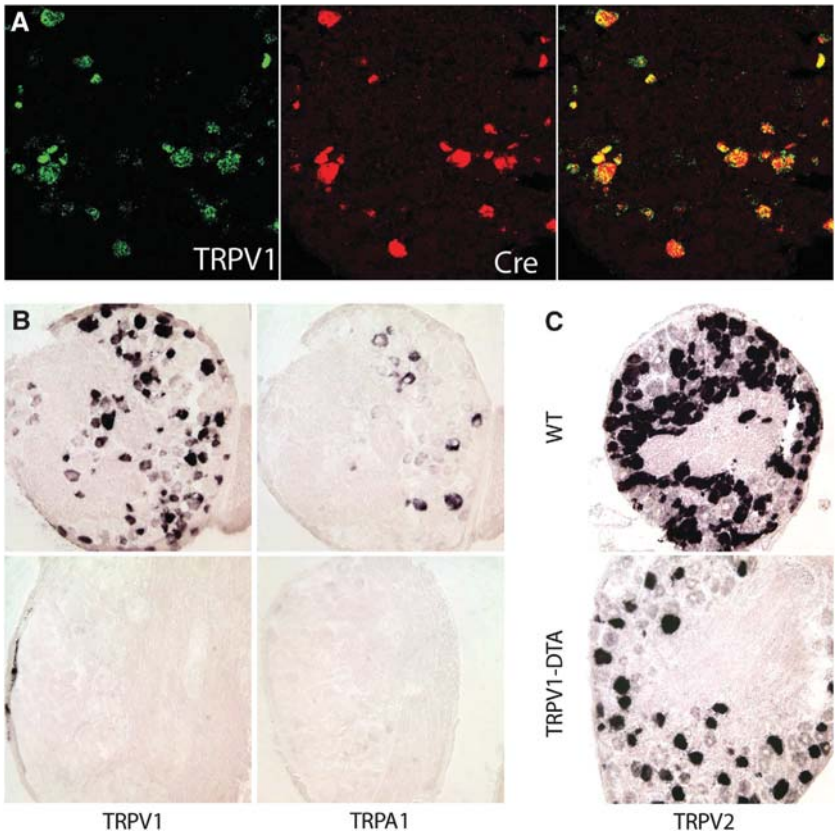


Figure 2 In adult mice, TRPV1-Cre is restricted to the TRPV1 neurons that are lost in TRPV1-DTA animals. Sections through the DRG of adult TRPV1-Cre mice were examined using *in situ* hybridization (**A**). Double labelling with probes for endogenous TRPV1 (green) and Cre (red) reveal complete co-expression (see merged image, right). (**B**) Staining for TRPV1 (left panels) and TRPA1 (right panels) demonstrate that >95% of positive neurons are lost in TRPV1-DTA mice (lower panels); in contrast (**C**) only a subset of TRPV2 cells are eliminated in these mutant animals.

indicated no noticeable differences between mutant and control animals (Supplementary Figure S3).

Figure 2B demonstrates that the TRPV1-DTA mice have lost all TRPV1- and TRPA1-expressing neurons in agreement with previous studies that demonstrate that TRPA1 is co-expressed in a subset of TRPV1 neurons (Story *et al*, 2003; Mishra and Hoon, 2010). Consistent with this, responses to capsaicin and mustard oil were completely abolished in standard eye wipe and paw injection paradigms (Supplementary Figure S4). Moreover, several well-characterized behavioural paradigms revealed that TRPV1-DTA mice were completely insensitive to noxious heat. For example, the mutant mice never reacted within the cutoff time when placed on a 55°C hot plate (Figure 3A) even after injection of carageenan to cause paw inflammation. TRPV1-DTA mice also failed to withdraw their tails (within the cutoff time) from radiant heat sources that burned the skin in a modified Hargreaves assay (Figure 3B) and most importantly showed no preference when given the choice between a 30°C platform and another at an elevated temperature (45 or 50°C) that normal mice strongly disliked (Figure 3C). Given the remarkable lack of protective thermosensory responses of the mutant animals, we also examined whether these mice had lost TRPV2- and other candidate thermosensory TRP-ion channels-expressing neurons using ISH (Figure 2C and Supplementary Figure S5). Although mutant animals showed a reduced number of TRPV2-containing sensory neurons, ~20% of positive cells remained, consistent with double labelling for TRPV1 and TRPV2 (data not shown). Finally, as predicted by their behavioural insensitivity to heat, there was also no heat-induced activation of *c-fos* in the dorsal horn of TRPV1-DTA mice (Figure 3D).

In marked contrast to their loss of heat sensation, TRPV1-DTA animals exhibited completely normal mechanosensory responses even after sensitization by inflammation or nerve ligation (Figure 4). We show that the behavioural responses of TRPV1-DTA and littermate controls are indistinguishable in assays for touch (Figure 4A and C) and pinch (Figure 4B). Importantly, recordings from the sciatic nerve to stimulation of the foot by brush, vibration and von Frey microfilaments were the same in TRPV1-DTA and control animals (Figure 4D and E). Moreover, the mutant mice displayed no loss of proprioceptive function for example, in rotarod assays (Figure 4F).

Cre-recombinase mediates DNA excision or rearrangement that is stable in all daughter cells. This can provide valuable information about development but means that recombination can occur in cells that no longer express Cre in the adult. In fact, TRPV1-ai9 animals appear to have many more tdTomato fluorescent neurons than expected based on TRPV1 (or Cre) ISH (compare Figures 1 and 2). Therefore, we carried out double label ISH for TRPV1 and tdTomato in sections through the ganglia of adult TRPV1-ai9 mice (Figure 5A). Approximately twice as many cells expressed the tdTomato as expressed TRPV1 or Cre, reflecting recombination

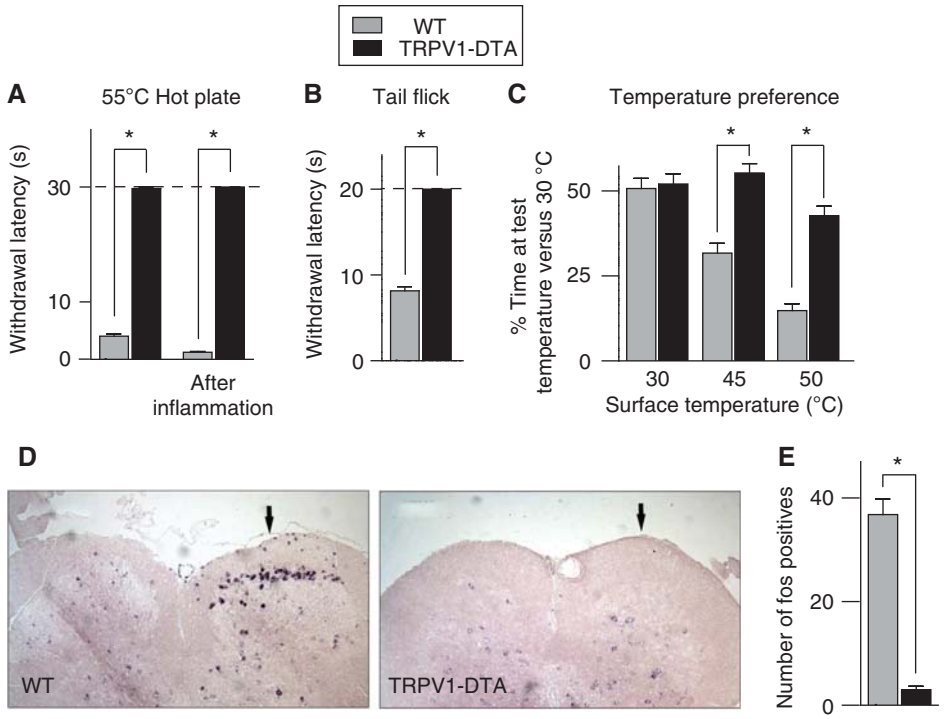


Figure 3 TRPV1-DTA mice lose all responses to heat. Compared with wild-type controls (grey bars), TRPV1-DTA mutant mice (black bars) exhibit no heat pain sensitivity in a hot-plate assay both before and after carageenan-induced inflammation (**A**) or in a tail-flick test (**B**) (*$P<0.0001$, Student's *t*-test); mutant mice did not react before the cutoff was reached in both tests (dotted line). Similarly, (**C**) a two-plate choice assays demonstrates that mutant animals fail to distinguish noxious (45 or 50°C) and normal (30°C) temperatures unlike normal animals (*$P<0.0001$, Student's *t*-test). Immersion of the paw in 55°C water (**D**, **E**) induces fos mRNA expression in the ipsilateral (arrowed) dorsal horn of wild-type but not in TRPV1-DTA mutant mice (**D**); fos-positive cell bodies in laminae I and II are quantified in (**E**) revealing a significant difference between genotypes (*$P<0.0001$, Student's *t*-test). Data represent means ± s.e.m.; $n \geqslant 6$ animals.

during development. Interestingly, double labelling showed that all TRPM8 cold sensing neurons expressed tdTomato (Figure 5B). Correspondingly, at early stages of embryonic development, most TRPM8-expressing neurons co-express TRPV1 (Supplementary Figure S2d) and this overlap disappears around birth (Supplementary Figure S2e and f). Therefore, we hypothesized that TRPM8 neurons would be ablated in TRPV1-DTA mice. Indeed, ISH (Figure 5C) shows that TRPV1-DTA mutants completely lack TRPM8-expressing neurons. These mice show no 'wet-dog shakes' to injection of icilin a compound that gives the percept of cooling and robustly induces this characteristic behaviour in control animals (Figure 5D). Notably, the mutant TRPV1-DTA mice also show absolutely no observable reaction to exposure to a cold plate at −5°C (Figure 5E) or preference for a 30°C environment over one at temperatures as low as 5°C in two choice preference assays (Figure 5F).

TRPM8-expressing cells contribute a relatively small proportion of the extra tdTomato-labelled neurons in the TRPV1-ai9 mice (Figure 5). Therefore, we examined other markers of somatosensory neurons that are not co-expressed with TRPV1 (Figure 6). TrkB and TrkC expression defines a class of completely non-overlapping cells but many of the Mrg neurons co-express tdTomato (Figure 6). Importantly, all tdTomato cells hybridize to a mixed probe including TRPV1, TRPM8 and several different Mrgs (Figure 6C) establishing that TRPV1-Cre-mediated recombination is limited to neurons expressing these markers. There is also a population of Mrg-expressing neurons that does not contain tdTomato in TRPV1-ai9 animals. Consistent with these findings, we observed that TRPV1-DTA animals retained 10–20% of the normal complement of Mrg-expressing neurons (Figure 6D).

Peripheral thermoreceptors and their role in setting body temperature

It has been shown that IP injection of capsaicin causes profound hypothermia in normal mice but not in TRPV1-KO animals (Caterina *et al*, 2000). We reasoned that ablation of hot and cold sensing neurons might dramatically alter thermoregulation and thus implanted internal temperature probes to measure core body temperature of TRPV1-DTA animals. The mutant animals exhibited a resting body temperature that was indistinguishable from that of littermate controls (Figure 7C) indicating that this temperature is maintained independent of peripheral somatosensory input. As expected, TRPV1-DTA animals lack responses to IP-injected capsaicin. As TRPA1 is expressed in a subset of the TRPV1-expressing neurons (Story *et al*, 2003; Mishra and Hoon, 2010), we also tested the effect of IP injection of mustard oil and found that it induced profound hypothermia in normal but not in the mutant mice suggesting that TRPA1-expressing TRPV1 neurons contribute to thermal homoeostasis. Notably, we also found that when environmental temperature was changed (e.g., elevated to 35°C or reduced, 4°C) TRPV1-DTA mutant animals were far less able to maintain their body temperature than wild-type controls (Figure 7D and E).

Given the homoeostatic deficit shown by TRPV1-DTA animals in response to thermal stimuli, we investigated whether responses to non-thermal stimuli that induce changes in body temperature were affected by TRPV1-

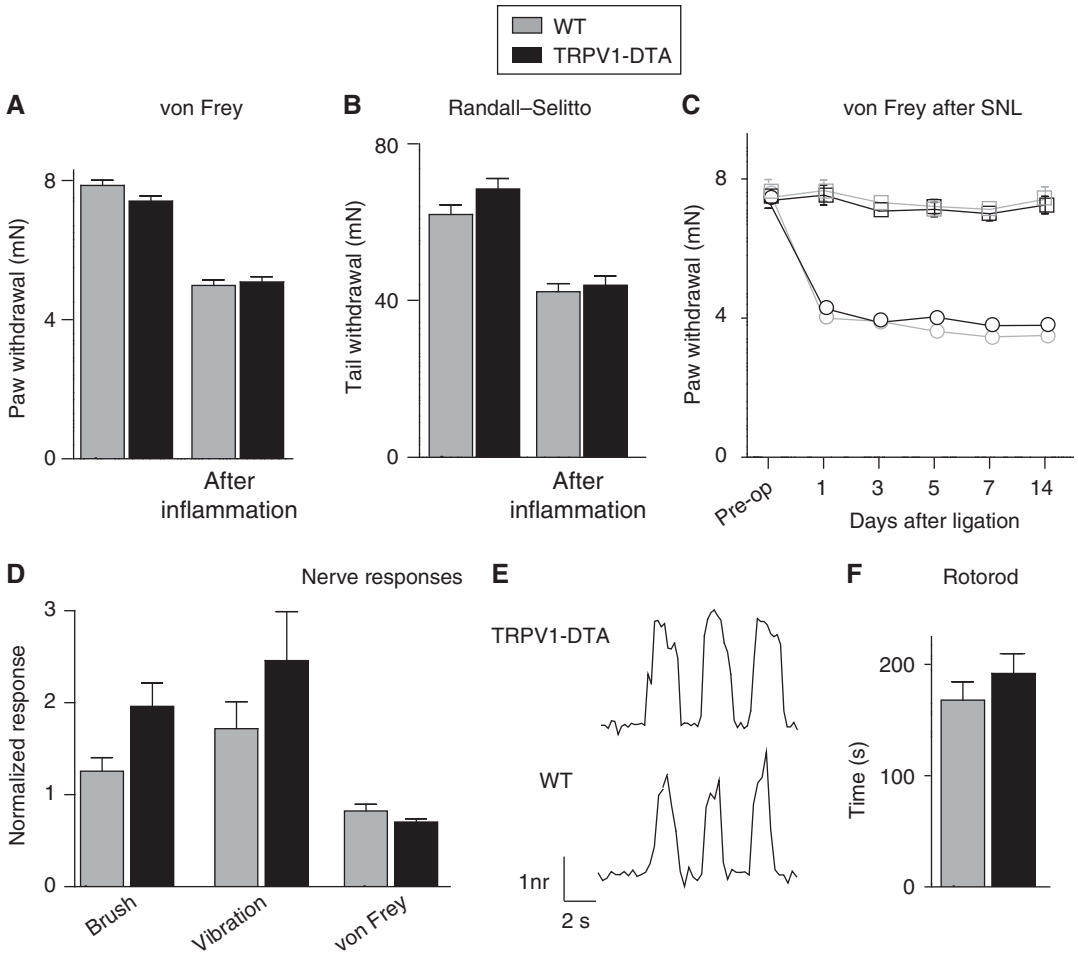


Figure 4 TRPV1-DTA mice retain normal touch, mechanical pain and proprioceptive responses. Measurements of touch and of mechanical pain following carageenan-induced inflammation (**A**) or sciatic nerve ligation (**C**) were made using von Frey microfilaments; mechanical pain was also assessed using a Randall–Selitto apparatus (**B**) both in normal and inflamed tissue. In addition, nerve recordings of the sciatic were measured in control and mutant mice (**D**) (responses were normalized to stimulation with a 6 g von Frey microfilament (nr)). Examples of summated responses to repeated 60 Hz vibrations are shown in (**E**). No significant difference was observed between wild-type and TRPV1-DTA mutant animals in any assay. Similarly, the two groups showed equivalent proprioception and motor function in a rotarod assay (**F**). Data represent means ± s.e.m.; $n \geqslant 5$ animals.

mediated cell ablation. Hypothermia is a hallmark of anaphylaxis caused by mast cell-induced vasodilation and loss of heat through the skin (Gilfillan *et al*, 2009). Normal mice become hypothermic upon injection of an antigen but relatively quickly re-establish normal resting temperature (Figure 7F). In contrast, TRPV1-DTA mutant mice had much deeper hypothermic response to antigen with delayed recovery. Fever is another pathophysiological thermal response that instead elevates body temperature. The cytokine IL1β mimics this pathway and induces a mild fever in normal mice (Komaki *et al*, 1992). Again we found that TRPV1-DTA animals exhibited more pronounced temperature change in response to IL1β than controls indicating that peripheral thermosensation has a role in reducing fever (Figure 7G).

TRPV1-DTA animals lose responses to painful and itch-inducing chemical stimuli

Many chemicals can induce pain when injected into tissue, among these is a class of noxious compounds that are believed to be released at sites of injury and include ATP, prostaglandins, bradykinin, histamine and serotonin (termed the 'inflammatory soup' (Kessler *et al*, 1992)). Receptors for the components of the inflammatory soup have been identified, but it has been unclear which classes of neurons are necessary for nociception. Therefore, we examined whether TRPV1-DTA mice responded to ATP or the whole 'inflammatory soup' and show (Figure 8A and B) that normal responses are completely lost in mutant animals. Thus, although purinergic receptors are expressed by neurons that are not ablated in the TRPV1-DTA mice (see Figure 8C), it appears that these cells are not involved in the noxious response to ATP or other components of the 'inflammatory soup' in the mutant. The simplest explanation is that these mice lack all nociceptors to this class of compounds. However, TRPV1 neurons express a variety of neuropeptides including CGRP and substance P, which have potential roles in neurogenic inflammation and sensitization of responses to the 'inflammatory soup'; their loss may dampen responses in other cells. Indeed, ISH (Figure 8C) demonstrated complete loss of substance P and almost total loss of CGRP in sensory ganglia from TRPV1-DTA mice predicting a significant loss in neurogenic inflammation. Measurements of swelling confirm that significantly less inflammation occurs in TRPV1-DTA animals challenged with carageenan (Figure 8D).

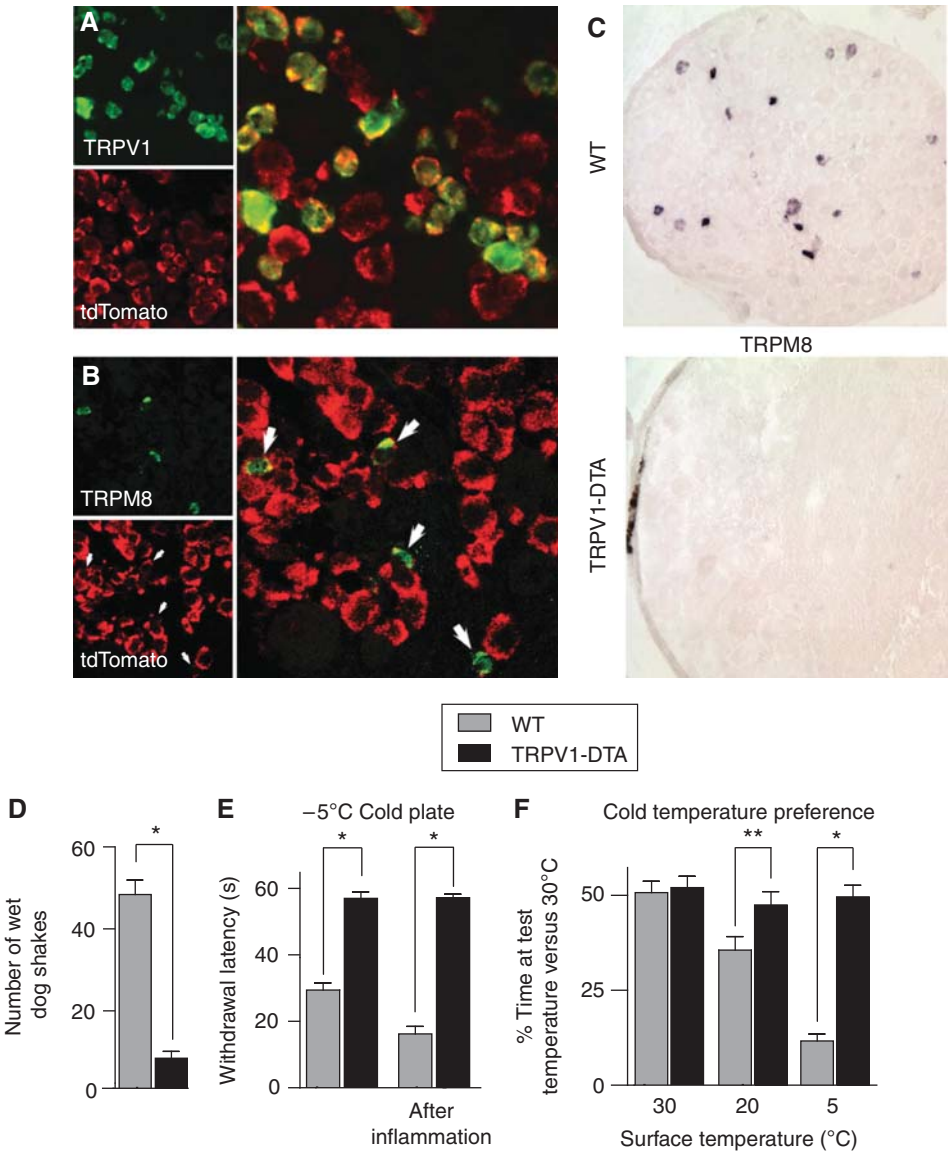


Figure 5 TRPV1-Cre mediates recombination in TRPM8 neurons and TRPV1-DTA mice lose cold sensation. Double-label ISH of sections through the DRG of TRPV1-ai9 mice (**A**, **B**) establish that recombination and tdTomato expression (red) occurs in more neurons than those expressing TRPV1 (**A**, green); all cells that express TRPM8 (**B**, green) also express tdTomato (cells co-expressing TRPM8 and tdTomato are arrowed). TRPV1-DTA mice lose all neurons expressing the TRPM8-cold receptor (**C**), and exhibit severely attenuated responses ('wet-dog shakes') to icilin (50 mg/kg) (**D**). In a cold-plate assay (**E**), mutant mice (black bars) failed to respond to −5°C even after carragenaan-induced inflammation. Moreover, unlike wild-type controls (grey bars) TRPV1-DTA mice (black bars) display no preference for warm (30°C) over a cooler (20°C) or cold (5°C) environments in long-term choice assays (**F**). Data represent means ± s.e.m.; $n \geqslant 6$ animals; significant differences between genotypes (Student's *t*-test) are indicated by $^{*}P < 0.0001$ and $^{**}P < 0.01$.

In addition to chemical, mechanical and thermal pain, somatosensory neurons transmit responses to pruritogenic agents that lead to the itch response (Shim and Oh, 2008). We found that TRPV1-DTA mutant mice had a complete loss of behavioural responses to subcutaneous injection of three major pruritogenic compounds: histamine, serotonin and a protease-activated receptor agonist (Figure 8E). Thus, TRPV1-DTA animals show profound deficits in response to many but not all nociceptive stimuli.

Discussion

In this study, we have used a molecular genetic approach to examine a defined subset of somatosensory neurons that express (or are derived from cells that express) a heat-sensitive ion channel by driving Cre-recombinase under the control of *TRPV1*. Our data strongly imply that TRPV1 expression is almost completely restricted to sensory neurons and a subset of cells lining blood vessels and/or their precursors. Strikingly, we see no evidence for TRPV1 expression in fungiform taste receptor cells (Figure 1I) contrary to the proposal that this channel mediates amiloride-insensitive salt taste responses (Lyall *et al*, 2004). Moreover, despite suggestions that hypothalamic TRPV1 might have a role in temperature homoeostasis (Mezey *et al*, 2000), this region of the brain was devoid of labelled cells (Figure 1G). In adult animals, all somatosensory neurons that express TRPV1 show Cre-mediated recombination. In addition, recombination selectively occurs in all TRPM8 cold-sensitive cells as well as a large subset of the Mrg-expressing neurons. These

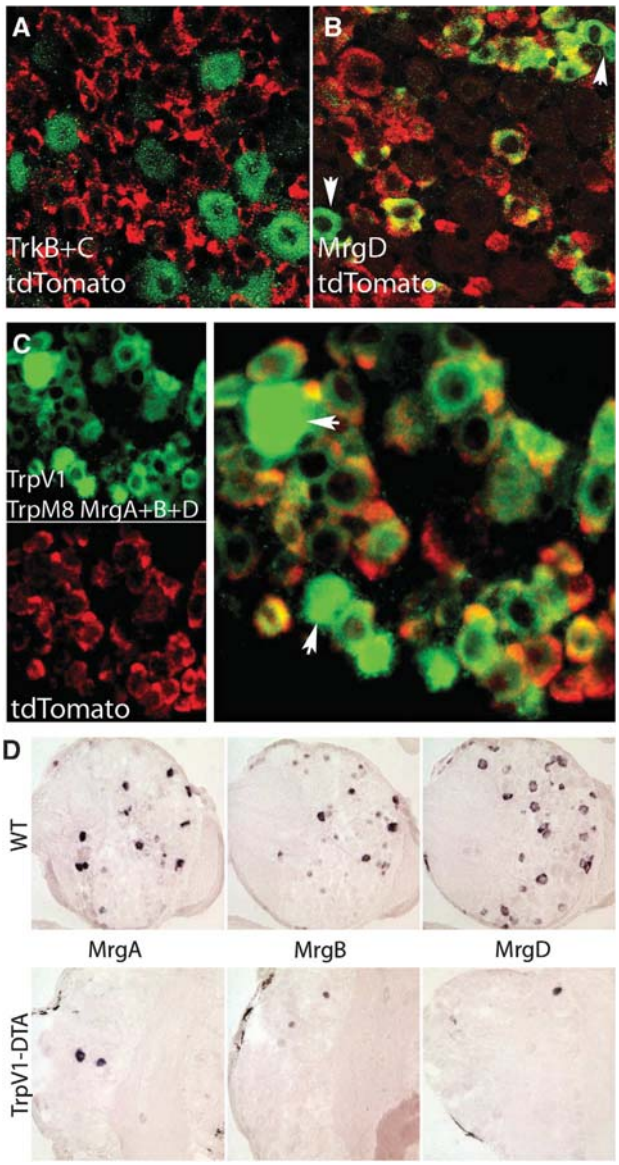


Figure 6 TRPV1-Cre recombination occurs in a restricted population of sensory neurons. The extent of TRPV1-Cre-mediated recombination in somatosensory neurons was assessed using ISH. Sections through the DRG of TRPV1-ai9 mice (**A–C**) were probed for Cre-mediated recombination using a tdTomato antisense probe (red). TdTomato is not expressed in TrkB- and C-neurons (green; **A**) but does mark most MrgD-expressing neurons (green and red, **B**); a few MrgD cells not co-llabeled by tdTomato are arrowed. A mixed antisense probe to TRPV1, TRPM8 and Mrgs (green, **C**) labels all cells in which recombination (and tdTomato expression, red) occurs. As expected from (**B**), there is a subset of cells that hybridize to the pooled probe that do not express tdTomato (arrowed in **C**). Single labelling for individual classes of Mrg (**D**) demonstrates that 80–90% of MrgA, MrgB and MrgD-expressing neurons are eliminated in TRPV1-DTA mice.

data confirm previous reports suggesting that TRPM8- and many Mrg-neurons are derived from embryonic cells that express TRPV1 (Hjerling-Leffler *et al*, 2007; Luo *et al*, 2007; Takashima *et al*, 2010). We used Cre-mediated expression of DTA to ablate this entire class of neurons and generated mice that were completely insensitive to hot or cold. In marked contrast, knockout of TRPV1 (Caterina *et al*, 1999) had almost no effect on detection of heat, and TRPM8 knockout mice (Bautista *et al*, 2007; Colburn *et al*, 2007; Dhaka *et al*, 2007) displayed only loss of cool but not noxious cold sensation. The TRPV1-DTA mutants also lacked responses to several noxious chemicals including components of the 'inflammatory soup' and pruritogenic agents. In addition, we show that although TRPV1-DTA mice normally maintain body temperature, they exhibit deficits in thermoregulation both in response to thermal and non-thermal challenges. Finally, our data reveal that DTA-mediated ablation of this large class of cells, including almost all the peptidergic neurons has no effect on mechanosensation.

TRPV1 is expressed in the development of many somatosensory neurons

Previous studies have shown that peptidergic TRPV1-, non-peptidergic Mrg- and the cold-sensitive TRPM8-expressing nociceptors are derived from common precursors whose development is under the control of the growth factor receptor TrkA (Chen *et al*, 2006; Luo *et al*, 2007; Shibasaki *et al*, 2010). It has been postulated that these precursor cells express TRPV1, as recordings from cultured embryonic cells show overlap in responses to TRPV1 and TRPM8 agonists (Hjerling-Leffler *et al*, 2007), and labelling studies indicate that many TRPV1-expressing neuron co-express TRPM8 (Takashima *et al*, 2010). Our data establish that TRPV1 is expressed early in the development of all TRPM8 neurons and a large subset (but not all) of Mrg neurons. However, because TRPV1-knockout mice do not exhibit major differences in the number or distribution of these classes of cell (Caterina *et al*, 2000), TRPV1 itself is not essential for their specification. In contrast to the expression of TrkA in presumptive nociceptors, TrkB and TrkC are believed to define mechanosensitive and proprioceptive neurons (Huang *et al*, 1999). We see no evidence for TRPV1-Cre expression in these neurons and correspondingly TRPV1-DTA animals show no discernible deficits in either mechanosensation or proprioception.

Recently, ablation of MrgD neurons in adult mice, but not throughout development, was reported to affect mechanosensation (Cavanaugh *et al*, 2009). Taken together with our data, these results indicate significant plasticity in the development of the mechanosensory system. Importantly, the restricted phenotype reported for mice lacking MrgD neurons suggests that this class of neurons is likely to have a very minor role in the behavioural deficits that we observe in TRPV1-DTA animals.

Although TRPV1-DTA mice have lost a large subset of nociceptors, including all TRPV1 cells, they retain a small population of neurons that express CGRP as well as neurons that express purinergic receptors (Figure 8). Interestingly, nociceptive responses to ATP injection are completely lost in these animals as are responses to a broader range of algesic mediators known as the 'inflammatory soup'. We suggest that a subset of the TRPV1-expressing neurons are likely responsible for painful reaction to all these compounds. Moreover, as subcutaneous injection of some of these agents (as well as additional compounds) causes itch and this is also lost in the TRPV1-DTA mutants, it seems likely that a subset of TRPV1 cells produce the puritogenic response. These data extend the recent proposals that TRPV1 and TRPV1-expressing neurons have a role in itch and that there are distinct labelled lines for itch and pain responses (Imamachi *et al*, 2009; Liu *et al*, 2009).

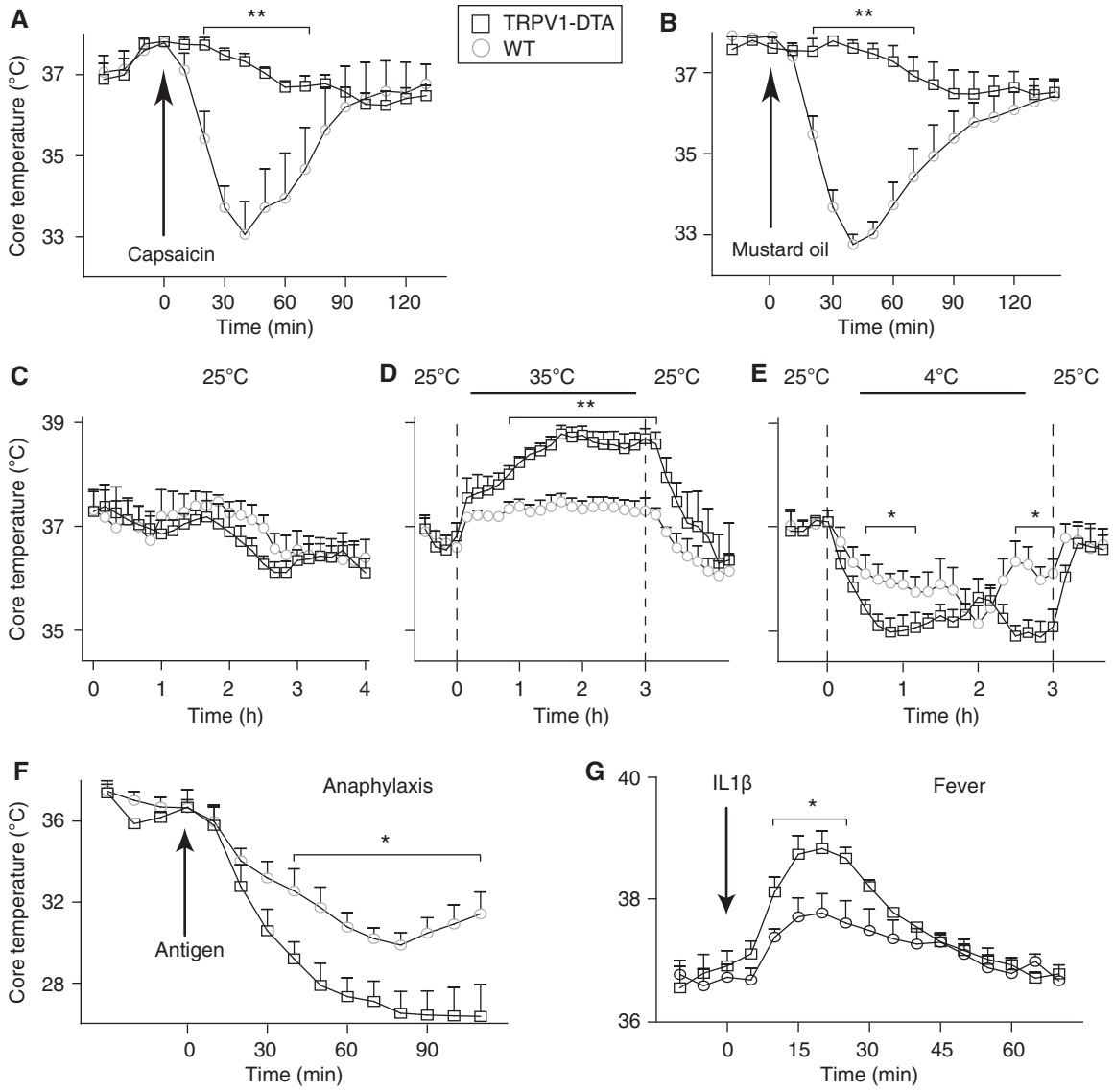


Figure 7 TRPV1-DTA mice show defective thermal homoeostasis. Implanted thermal sensors were used to study the core temperature of TRPV1-DTA (□) and wild-type control animals (○). Control animals exhibit pronounced hypothermia following injection of 20 μg of capsaicin (**A**) or 0.5 μg of mustard oil (**B**), but as expected, TRPV1-DTA mutant mice are completely unresponsive to these chemicals. Although mutant animals maintain normal body temperature at 25°C (**C**), they show defective control of temperature homoeostasis following exposure to increased (35°C, **D**) and decreased (4°C, **E**) environmental temperatures. Non-thermally induced changes in body temperature are also more pronounced in TRPV1-DTAs than in control animals; for example, temperature decline following anaphylaxis (**F**) and IL1β-induced fever (**G**) are increased in the mutant mice. Data represent means ± s.d.; $n = 5$–6 animals, significant differences between genotypes (Student's *t*-test) are indicated by $^{**}P<0.01$ and $^{*}P<0.05$.

The receptors and cells for thermosensation

Several different TRP channels are believed to provide animals with a molecular thermometer (Jordt *et al*, 2003; Patapoutian *et al*, 2003) with different TRPs activated over distinct temperature ranges. Of these, the best characterized are TRPV1, inactive below 42°C, (Caterina *et al*, 1997) and TRPM8, inactive above 25°C (McKemy *et al*, 2002; Peier *et al*, 2002). In addition TRPV2, TRPV3, TRPV4 and TRPA1 have been proposed as additional and differentially tuned sensors (Jordt *et al*, 2003; Patapoutian *et al*, 2003; Karashima *et al*, 2009; Knowlton *et al*, 2010). Interestingly, elimination of TRPV1 (Caterina *et al*, 2000), TRPV3 (Moqrich *et al*, 2005), TRPV4 (Lee *et al*, 2005) and TRPA1 (Bautista *et al*, 2006; Kwan *et al*, 2006) have only very modest effects on thermosensation, while TRPM8 knockout animals (Bautista *et al*, 2007; Colburn *et al*, 2007; Dhaka *et al*, 2007) exhibit a reduction in their responses to cool <20°C but not cold <5°C. By contrast, animals lacking the cells expressing TRPV1, TRPA1 and TRPM8 have no response to either hot or cold. As pharmocological silencing of the TRPV1 sensory input (Cavanaugh *et al*, 2009), which includes the TRPA1 cells (Story *et al*, 2003), affects hot but not cold responses we conclude that TRPM8 cells carry much of the critical cold sensory information; that is, there are separate hot- and cold-labelled lines.

The mild phenotypes of TRPV1, TRPA1 and TRPM8 knockout animals (Caterina *et al*, 2000; Bautista *et al*, 2006, 2007; Kwan *et al*, 2006; Colburn *et al*, 2007; Dhaka *et al*, 2007) suggest that there might be additional thermosensory receptors including TRPV2, TRPV3 and TRPV4 (Caterina *et al*,

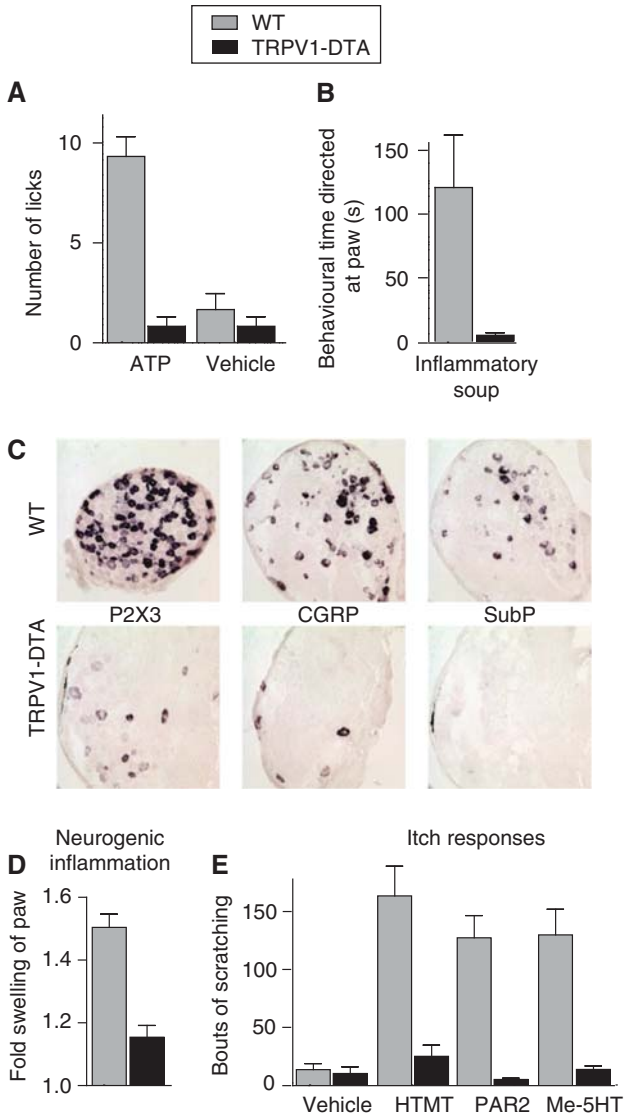


Figure 8 TRPV1-DTA mice lack algesic and pruritogenic chemical responses. Control mice (grey bars) exhibit pronounced and stereotypic pain responses to intraplantar injection of ATP (**A**) or a mix of agents known as the 'inflammatory soup' (**B**). In contrast, TRPV1-DTA mice (black bars) do not react to these chemicals (**A**, **B**). *In situ* hybridization of sections through the DRG (**C**) demonstrates that TRPV1-DTA mice have lost the majority (but not all) of the neurons expressing the purinergic receptor P2X3 and the neuropeptide CGRP as well as all cells expressing substance P (SubP). Mutant mice exhibit a decrease in neurogenic inflammation as measured by paw swelling in response to carageenan injection (**D**). In addition, TRPV1-DTA animals are much less sensitive to sub-dermal injection of a variety of compounds that induce itch than normal control mice (**E**). Data are mean ± s.e.m.; $n = 6$ animals; significant differences between genotypes (Student's *t*-test) were $P < 0.0001$ for responses to ATP, inflammatory soup and pruritogenic compound and $P < 0.01$ for neurogenic inflammation.

1999; Lee *et al*, 2005; Moqrich *et al*, 2005). Our data demonstrate that sensory input through any such proteins must be missing in TRPV1-DTA animals and because many neurons still express TRPV2 in TRPV1-DTA mice (Figure 2), we suggest that TRPV2 probably has a non-thermosensory role.

Why do the knockouts of individual TRP channels have such a minor effect on behaviour, when our data from TRPV1-DTA animals and recent reports using pharmacological agents (Cavanaugh *et al*, 2009; Mishra and Hoon, 2010) suggest that the cells expressing these channels are crucial for detecting hot and cold? Given the large number of TRP-related and other potential thermosensors (Jordt *et al*, 2003; Patapoutian *et al*, 2003), it is likely that some redundancy accounts for this difference. However, the complete loss of both hot and cold responses in TRPV1-DTA mutants shows that only a limited subset of somatosensory neurons conveys thermal information.

Peripheral thermosensation and the regulation of body temperature

Capsaicin rapidly induces hypothermia in normal, but not TRPV1-knockout mice showing that activation of TRPV1 affects temperature homoeostasis. Thus, it has been suggested that TRPV1-expressing neurons provide the central control circuits in the hypothalamus with information about the temperature of the periphery (Caterina *et al*, 2000). It should be noted that although this hypothesis is attractive and likely substantially correct, it has not yet been formally

possible to rule out peripheral effects in the response to capsaicin; for example, systemic activation of TRPV1 neurons may result in release of neuropeptides that affect body temperature through vasodilation. Regardless of the exact mechanism, recent data showing that TRPV1 antagonists produce hyperthermic responses (Gavva *et al*, 2008) demonstrate that some TRPV1 fibres and TRPV1 channels must be active at normal body temperatures. The TRPV1-DTA mice that we describe in this study significantly extend our knowledge of the role of peripheral thermosensation in control of body temperature. Notably, TRPV1-DTA mutants generally maintain a standard body temperature revealing that additional (presumably central) thermosensors must have the major homoeostatic function. However, these mice exhibit exaggerated fluctuations when placed under various types of thermal and non-thermal stress suggesting peripheral sensory neurons may have a role in feedback control (see Figure 7). In contrast, TRPV1-knockout mice were found to have nearly wild-type temperature homoeostasis in response to similar challenges (Iida *et al*, 2005). Thus, just as in temperature sensing, our data studying body temperature reveal that TRPV1 expression marks the neurons that are crucial for detecting temperature throughout the periphery and that TRPV1 is likely to only be one of several thermosensors that activate the neurons.

Concluding remarks

In this study, we have engineered mice that help establish the selectivity of TRPV1 expression primarily in a subset of somatosensory neurons. TRPV1 is an early developmental marker of all the hot- and cold-sensing neurons meaning that TRPV1-DTA mice are completely insensitive to their environmental temperature. Nonetheless, these mutant animals exhibit perfectly normal mechanical responses and proprioception, highlighting that sensory neurons are specified to respond to distinct modalities even where their activation ultimately results in the common sensation of pain.

Materials and methods

Animal models

Mice were 20–30 g (2- to 4-month old) TRPV1-cre, Rosa-DTA (Ivanova *et al*, 2005) or Rosa-tdTomato (Madisen *et al*, 2010) and were intercrossed to generate experimental animals as described in the text; the TRPV1-Cre was hemizygous and knock-in backgrounds heterozygous. The TRPV1-IRES-Cre construct was generated by recombination as described previously (Lee *et al*, 2001) using the RP23-181P10 BAC, importantly a region in the last exon was deleted from the transgene that included the last 20 codons and all the predicted 3′ UTR (this fragment was used to probe for native transcript by ISH). Inflammation was induced in the plantar surface of a hind paw or the tail by injection of 20 μl of a 2% carageenan solution (Sigma) in PBS. We monitored inflammation in animals using a plethysmometer device (IITC Life Science, USA). In a model of neuropathic pain, a partial nerve ligation was made by tying a tight ligature around the sciatic nerve (Seltzer *et al*, 1990). Core body temperature was measured using intraperitoneal implanted telemetric temperature probes (Data Sciences Inc.). Incisions were made through the skin and probe inserted under the abdominal wall. Experiments were initiated more than 1 week after surgery. Procedures followed the NIH Guidelines for the care and use of laboratory animals, and were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee.

Behavioural assays

Chemical sensitivity. Eye-wipe assays were performed to investigate the afferent functions of the ophthalmic branch of the trigeminal nerve. Capsaicin and mustard oil-induced eye wipes were counted for 1 min after delivery of 50 μl of solution (100 μM capsaicin or 10 mM mustard oil in PBS). We chose this concentration of mustard oil because it has been shown to have no effect on TRPA1-KO mice (Bautista *et al*, 2006; Kwan *et al*, 2006; Mishra and Hoon, 2010); that is, it is completely TRPA1 specific and does not activate other receptors including TRPV1. Peripheral responses to capsaicin (1 μg) and mustard oil (10%) injection (10 μl) in the hind paw were recorded as described in (Caterina *et al*, 2000). Wet-dog shakes were induced by i.p. injection of 50 mg/kg icilin (Sigma) and number of whole-body shakes counted over 30 min as described (Dhaka *et al*, 2007).

Thermal responses. We used a semi-automated tail-flick test (IITC Life Science) to measure thermal responses. Animals were habituated for 10 min in individual chambers before experiments. A radiant heat source was focused on the tail, and the time from the initiation of the radiant heat until withdrawal was measured. A maximum cutoff of 20 s was used to prevent tissue damage. Hot/cold-plate test was used to assess acute temperature sensitivity, mice were placed on single hot plate at 55°C or −5°C and latency to display withdrawal of hind limbs was measured. Cutoffs were set at 30 s (55°C) and 1 min (−5°C) to prevent tissue damage. Two choice temperature preference/aversion assays were employed to determine thermal thresholds; mice were placed in an apparatus that had plates at 30°C and a variable test temperature (T2CT, Bioseb, France). Assays were initiated by placement at the test temperature; each animal was tested twice wherein the order of initial placement was reversed. Mouse position was tracked over 5 min; to ensure data were collected from trials in which animals were exposed to both test temperature and 30°C, only assays in which mice sampled both temperatures in the first minute were counted.

Mechanical responses. Threshold-force-induced paw withdrawal was measured using an automated von Frey apparatus (Ugo Basile Varese, Italy); in addition, a modified Randall–Selito device (IITC Life Sciences) was used to automatically measure responses when pressure was applied to the tail.

Sciatic nerve recordings. Mice were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). A deep level of anaesthesia was maintained throughout the experiment using additional sodium pentobarbital, and body temperature was maintained between 35 and 37°C. Briefly, the common peroneal branch of the sciatic nerve was accessed through an incision in the lateral side of the left thigh. To expose the nerve, the biceps femoris was cut. The nerve was then dissected free from surrounding tissue, cut near the branch point from the rest of the sciatic nerve and placed on a (2–4 MEG) tungsten microelectrode (Frederick Haer & Co). A reference electrode was placed in nearby muscle tissue. The incision cavity was then filled with halocarbon oil (Sigma) to slow fibre desiccation. Digitization of nerve responses was performed using Clampex 10.2 of the pClamp software package (Molecular Devices, Sunnyvale, CA), running on an IBM-compatible computer. All responses were amplified using a Grass P511AC amplifier (Grass Technologies, West Warwick, RI) and digitized with Digidata 1322A (Molecular Devices). Data were analyzed using Matlab 7 (Mathworks, Lyme, NH). Three types of stimuli were used to describe mechanical touch sensation on the dorsal side of the foot: brush, 60 Hz vibration and von Frey filaments (4 and 6 g). Animals were tested with individual stimuli three times and these tests were repeated three times, response maxima (versus baseline) were calculated and normalized against the average response from a 6 g von Frey stimuli. Assessment of proprioceptive and motor performance was performed on an accelerating rotarod (4–40 r.p.m. in 5 min; Med Assoc. Inc.). Hypothermia was induced by i.p. injection of 20 μg of capsaicin or 0.5 μg of mustard oil (in 20 μl of saline). The dose of mustard oil in this experiment is ∼100 lower than that shown to be selective for TRPA1 (see above eye-wipe assays). Initially, following IP injection the mice are quite different, capsaicin treatment leads to a short period of intense agitation and nociferous behaviour directed to the site of injection, mustard oil injected mice in contrast show much less reaction (possibly because

such a low dose was used). During the period when mice are hypothermic both treatments result in reduced movement and a curling-up behaviour that are indistinguishable. This is consistent with mustard oil activating a different receptor and cell type than capsaicin.

Anaphylaxis. Mice were sensitized by i.v. injection of 20 μg mouse mAb IgE anti-dinitrophenol (DNP) and 20 h later, anaphylaxis was induced by i.v. injection of 1 mg DNP-coupled human serum albumin (Sigma). Temperature was monitored for up to 2 h after which mice were euthanized.

Fever. Recombinant IL1β (R & D Systems) was administered by i.v. injection (100 ng).

Scratching behaviour. Hair was removed from the nape of the neck, and pruritogens administered by 10 μl subcutaneous injection (500 nMol histamine trifluoromethyl toluidide (HTMT; Tocris), 100 μg SLIGRL-NH2 (Tocris), 30 μg Methyl-serotonin (Me-5HT; Sigma)) and PBS was used as a control. Bouts of scratching were counted over a 30-min observation period.

Algesic substances. Numbers of flinches, guarding and licks were monitored over 10 min after intraplantar injection of 10 μl of ATP (500 nmol), or inflammatory soup (serotonin, histamine, PGE2 and bradykinin; 1 μmol each). PBS was used as a control and vehicle.

In situ hybridization

ISH was performed at high stringency (washed 30 min, 0.2 × SSC, 70°C) as described previously (Hoon *et al*, 1999). ISH of molecular markers was performed on tissue from >10 TRPV1-DTA and control mice. Serial sections from >10 sections per mouse were hybridized and numbers of cells counted in order to quantize ablation of neurons.

Fos expression. Mice were anaesthetized with avertin (2.5% 20 ml/kg) before immersion of hind paw in water at 55°C, three times for 30 s each with a 1 min interstimulus interval; 30 min after thermal challenge, mice were euthanized and L4/5 spinal cords harvested. ISH was performed with fos riboprobe on tissue taken from three control and TRPV1-DTA animals; counts of fos-positive stained cells were made on 10 sections per animal.

For double-label ISH, we used fluorescein- and digoxigenin-labelled probes that were detected with antibodies coupled to horseradish peroxidase and alkaline phosphatase, respectively, together with tyramide–FITC and fast-red (Adler *et al*, 2000). Images were collected using a Microphot FX microscope (Nikon) and confocal microscopy (1 μm optical sections) was with a Leica TCS SP2 (Leica Microsystems) and images were processed with Adobe Photoshop.

Gene array analysis

Total RNA was isolated from DRG using an RNeasy minikit (Qiagen), cDNA was produced, and amplified cRNA prepared as described by the manufacturer (one-step probe synthesis kit; Agilent). Gene arrays (GE 4 X 44K V2; Agilent) were hybridized, washed and scanned as recommended by the manufacturer. Data were analyzed using Gene Spring (version 11) software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Dr Lars von Buchholtz for the taste data and review of the manuscript. We are also very grateful to Nick Ryba for encouragement and helpful advice and Drs Nguyen, Usdin, Gutkind and Siraganian for valuable suggestions. Transgenic mice were generated by Andrew Cho in the NIDCR-core. This research was supported by the intramural research programme of the NIH, NIDCR (MAH).

Author Contributions: SKM carried out and analyzed the majority of the experiments, SMT and PO contributed data for Figures 8 and 4 respectively, SKB data from nerve ligation studies and MAH designed the study, generated and analyzed data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abrahamsen B, Zhao J, Asante CO, Cendan CM, Marsh S, Martinez-Barbera JP, Nassar MA, Dickenson AH, Wood JN (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. *Science* **321:** 702–705

Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, Zuker CS (2000) A novel family of mammalian taste receptors. *Cell* **100:** 693–702

Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D (2006) TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* **124:** 1269–1282

Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* **448:** 204–208

Cain DM, Khasabov SG, Simone DA (2001) Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an *in vivo* study. *J Neurophysiol* **85:** 1561–1574

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288:** 306–313

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* **398:** 436–441

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389:** 816–824

Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc Natl Acad Sci USA* **106:** 9075–9080

Chen CL, Broom DC, Liu Y, de Nooij JC, Li Z, Cen C, Samad OA, Jessell TM, Woolf CJ, Ma Q (2006) Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. *Neuron* **49:** 365–377

Colburn RW, Lubin ML, Stone Jr DJ, Wang Y, Lawrence D, D'Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N (2007) Attenuated cold sensitivity in TRPM8 null mice. *Neuron* **54:** 379–386

Dhaka A, Murray AN, Mathur J, Earley TJ, Petrus MJ, Patapoutian A (2007) TRPM8 is required for cold sensation in mice. *Neuron* **54:** 371–378

Gavva NR, Treanor JJ, Garami A, Fang L, Surapaneni S, Akrami A, Alvarez F, Bak A, Darling M, Gore A, Jang GR, Kesslak JP, Ni L, Norman MH, Palluconi G, Rose MJ, Salfi M, Tan E, Romanovsky AA, Banfield C *et al* (2008) Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* **136:** 202–210

Gilfillan AM, Peavy RD, Metcalfe DD (2009) Amplification mechanisms for the enhancement of antigen-mediated mast cell activation. *Immunol Res* **43:** 15–24

Hjerling-Leffler J, Alqatari M, Ernfors P, Koltzenburg M (2007) Emergence of functional sensory subtypes as defined by transient receptor potential channel expression. *J Neurosci* **27:** 2435–2443

Hoon MA, Adler E, Lindemeier J, Battey JF, Ryba NJ, Zuker CS (1999) Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell* **96:** 541–551

Huang EJ, Wilkinson GA, Farinas I, Backus C, Zang K, Wong SL, Reichardt LF (1999) Expression of Trk receptors in the developing

mouse trigeminal ganglion: *in vivo* evidence for NT-3 activation of TrkA and TrkB in addition to TrkC. *Development* **126:** 2191–2203

Iida T, Shimizu I, Nealen ML, Campbell A, Caterina M (2005) Attenuated fever response in mice lacking TRPV1. *Neurosci Lett* **378:** 28–33

Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, Han SK (2009) TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc Natl Acad Sci USA* **106:** 11330–11335

Ivanova A, Signore M, Caro N, Greene ND, Copp AJ, Martinez-Barbera JP (2005) *In vivo* genetic ablation by Cre-mediated expression of diphtheria toxin fragment A. *Genesis* **43:** 129–135

Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Hogestatt ED, Meng ID, Julius D (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427:** 260–265

Jordt SE, McKemy DD, Julius D (2003) Lessons from peppers and peppermint: the molecular logic of thermosensation. *Curr Opin Neurobiol* **13:** 487–492

Karashima Y, Talavera K, Everaerts W, Janssens A, Kwan KY, Vennekens R, Nilius B, Voets T (2009) TRPA1 acts as a cold sensor *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* **106:** 1273–1278

Kessler W, Kirchhoff C, Reeh PW, Handwerker HO (1992) Excitation of cutaneous afferent nerve endings *in vitro* by a combination of inflammatory mediators and conditioning effect of substance P. *Exp Brain Res* **91:** 467–476

Knowlton WM, Bifolck-Fisher A, Bautista DM, McKemy DD (2010) TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics *in vivo*. *Pain* **150:** 340–350

Komaki G, Arimura A, Koves K (1992) Effect of intravenous injection of IL-1 beta on PGE2 levels in several brain areas as determined by microdialysis. *Am J Physiol* **262**(2 Part 1)**:** E246–E251

Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP (2006) TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* **50:** 277–289

Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG (2001) A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73:** 56–65

Lee H, Caterina MJ (2005) TRPV channels as thermosensory receptors in epithelial cells. *Pflugers Arch* **451:** 160–167

Lee H, Iida T, Mizuno A, Suzuki M, Caterina MJ (2005) Altered thermal selection behavior in mice lacking transient receptor potential vanilloid 4. *J Neurosci* **25:** 1304–1310

Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng HJ, Geng Y, Undem BJ, Kollarik M, Chen ZF, Anderson DJ, Dong X (2009) Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. *Cell* **139:** 1353–1365

Luo W, Wickramasinghe SR, Savitt JM, Griffin JW, Dawson TM, Ginty DD (2007) A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. *Neuron* **54:** 739–754

Lyall V, Heck GL, Vinnikova AK, Ghosh S, Phan TH, Alam RI, Russell OF, Malik SA, Bigbee JW, DeSimone JA (2004) The mammalian amiloride-insensitive non-specific salt taste receptor is a vanilloid receptor-1 variant. *J Physiol* **558**(Part 1)**:** 147–159

Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13:** 133–140

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416:** 52–58

Mezey E, Toth ZE, Cortright DN, Arzubi MK, Krause JE, Elde R, Guo A, Blumberg PM, Szallasi A (2000) Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc Natl Acad Sci USA* **97:** 3655–3660

Mishra SK, Hoon MA (2010) Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. *Mol Cell Neurosci* **43:** 157–163

Moqrich A, Hwang SW, Earley TJ, Petrus MJ, Murray AN, Spencer KS, Andahazy M, Story GM, Patapoutian A (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science* **307:** 1468–1472

Mueller KL, Hoon MA, Erlenbach I, Chandrashekar J, Zuker CS, Ryba NJ (2005) The receptors and coding logic for bitter taste. *Nature* **434:** 225–229

Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. *Nat Rev Neurosci* **4:** 529–539

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002) A TRP channel that senses cold stimuli and menthol. *Cell* **108:** 705–715

Seltzer Z, Dubner R, Shir Y (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* **43:** 205–218

Shibasaki K, Murayama N, Ono K, Ishizaki Y, Tominaga M (2010) TRPV2 enhances axon outgrowth through its activation by membrane stretch in developing sensory and motor neurons. *J Neurosci* **30:** 4601–4612

Shim WS, Oh U (2008) Histamine-induced itch and its relationship with pain. *Mol Pain* **4:** 29

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112:** 819–829

Takashima Y, Ma L, McKemy DD (2010) The development of peripheral cold neural circuits based on TRPM8 expression. *Neuroscience* **169:** 828–842

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21:** 531–543

Wall PD, Devor M, Inbal R, Scadding JW, Schonfeld D, Seltzer Z, Tomkiewicz MM (1979) Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. *Pain* **7:** 103–111